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(51) International Patent Classification <sup>6</sup> :  C12N 15/85, A61K 48/00, C12N 15/52, 15/53, 9/00, 9/02	A2	(11) International Publication Number: <b>WO 99/60142</b>  (43) International Publication Date:    25 November 1999 (25.11.99)
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*Without international search report and to be republished upon receipt of that report.*

Vector material useful for antitumour therapy contains: (a) a tumour cell sensitizing gene or genes of which expression in a tumour cell yields a sensitizing gene expression product having a potential to cause tumour cells to be killed and destroyed, or to be eliminated, or otherwise to be inactivated, or to be rendered sensitive and/or vulnerable to destruction; (b) a sensitizing gene promoter; (c) at least one control gene; and (d) a control gene expression regulatory system responsive in use in a transduced cell to the effect of a predetermined exogenous or endogenous expression inducing influence, e.g. ionizing radiation, heat or a chemical inducing agent, so as to induce expression of the control gene to yield an expression product having a capacity to establish an operative linkage between the sensitizing gene promoter and the sensitizing gene or genes effective to trigger and switch on or permit continuous or permanent expression of the latter to bring about continuous production of the sensitizing gene expression product. This is preferably achieved by arranging for the control gene to encode a recombinase enzyme that acts on recombinase target sites in a Cre-loxP or Flp-frt site specific recombination system to remove an expression preventing stop cassette sequence between the sensitizing gene(s) and the promoter for the latter. In some embodiments the tumour sensitizing gene expression product will be an enzyme or other bioactive agent that can activate an inactive prodrug.

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GENE THERAPY VECTORS AND THEIR USE IN ANTITUMOUR  
THERAPY

FIELD OF THE INVENTION

The present invention relates to the field of molecular biology and  
5 gene therapy, especially as applied to cancer therapy.

BACKGROUND

In connection with cancer therapy it has been suggested that cancer  
cells may be effectively treated by introducing into them sensitizing foreign  
genes, the expression of which leads to the destruction or elimination of these  
10 cells. This could be achieved, for example, via the expression of a cytotoxic  
protein or cytotoxic RNA species, or via the expression of an immune-response  
stimulating factor or of substances that can bring about or promote the bio-  
conversion and activation of a systemically applied inactive chemical agent or  
prodrug to form an active cytotoxic drug (see for example Elizabeth A. Austin,  
15 *et al.*, (1992), "A First Step in the Development of Gene Therapy for Colorectal  
Carcinoma: Cloning, Sequencing, and Expression of *Escherichia coli* Cytosine  
Deaminase", *Molecular Pharmacology*, 43, 380-387). The latter approach has  
been generally favoured in most cases because the active drug so produced is  
able to kill cells in the vicinity of the sensitised activating cells (the "bystander"  
20 effect), thus compensating for any inefficiencies in cellular uptake of the  
activating gene expression system.

Difficulties with these gene therapy methods, however, include the fact  
that most current DNA or gene delivery and transfection systems usually  
propose the use of genetically engineered viruses which, up to now, have not  
25 been able to deliver DNA for therapeutic purposes exclusively to tumour cells.  
Although there have been attempts to circumvent this problem by employing  
expression controlling regions (promoter and/or enhancer elements) of genes

that are predominantly expressed in tumour cells to direct the expression of the tumour-cell sensitizing mechanism, it has had to be accepted that such genes can also be expressed in normal tissues so that selectivity of expression is not absolute. Methods for the delivery of the genes specifically to target tumour cells by exploiting cell-specific surface antibodies or receptors have also been considered, but these are also expressed in a number of normal cell types and thus selection is again not absolute. In order to be useful and safe for the treatment of cancer, ideally there needs to be a level or levels of selection that will result in expression of the cytotoxic mechanism in the tumour or in the vicinity of the tumour or in the tumour cells exclusively.

Some methods of cancer treatment depend on ionizing radiation which is very commonly used against a variety of cancers. Devices are widely available for directing the radiation from an external source to the tumour in such a way that the dose of radiation to normal tissues is minimised (conformal radiotherapy). However, problems associated with radiotherapy include the fact that tumour cells can often be more resistant to treatment than normal cells, whilst some normal cell types inside the radiation field may be very radiosensitive. In some alternative attempts to deliver the radiation more specifically only to tumour cells, tumour-targeting antibodies or similar molecules have been labelled with various radioisotopes. In this latter case, however, the amounts of radiation that can be administered overall to the tumour within tolerable systemic levels are often too small to be of sufficient direct therapeutic value and this approach has therefore been generally considered more suitable for tumour imaging than for therapy.

The term "ionizing radiation" as used herein may include not only electromagnetic radiation such as X-rays and  $\gamma$ -rays, but also high energy sub-atomic particles such as  $\alpha$ -particles and  $\beta$ -rays or electron beams.

Apart from chemotherapy, another method of cancer treatment uses diathermy. However, despite extensive trials, hitherto this method has not found widespread use because the response curves for human cells are very steep and because of difficulties in achieving and maintaining precise and  
5 homogeneous elevated temperatures in deep-seated tumours.

One objective of the present invention is to provide improved means and methods for selectively killing or eliminating tumour cells using a low or transient dose of a gene expression inducing agent to switch on a gene that produces an expression product within tumour tissue that has the effect of  
10 bringing about the destruction or removal of tumour cells.

#### SUMMARY OF THE INVENTION

The present invention relies at least in part on a concept of selectively bringing about the destruction or elimination of tumour cells by using gene therapy methods to provide the tumour cells with a silenced or dormant killing  
15 mechanism that can be activated by exposing the cells to an appropriate stimulating influence, e.g. ionizing radiation, heat, or a chemical or other inducing agent, so as to upregulate a control gene which then triggers and switches on or primes a tumour cell sensitizing gene or genes. It is in fact already known that expression of certain genes in human cells can be induced  
20 or upregulated by exposure of the cells to heat (e.g. from diathermic devices) or ionizing radiation, in the latter case sometimes after very low doses (see for example D.E. Hallahan *et al.* (1995) "Spatial and temporal control of gene therapy using ionizing radiation", *Nature Medicine*, Vol. 1, No. 8, 786-791) and D. E. Hallahan, *et al.*, (1995), "*c-jun* and *Egr-1* Participate in DNA Synthesis and Cell Survival in Response to Ionizing Radiation Exposure", *The Journal of Biological Chemistry*, 270, 30303-30309), but it is believed that this effect has  
25 not previously been exploited in the same way as in the present invention.

More specifically, the invention envisages providing means for carrying out a method of treatment for cancer patients in which there are delivered to tumour cells vectors, preferably self-replicating viral or plasmid vectors, containing a dormant or silenced tumour cell sensitizing gene or genes  
5 of which transcription and expression can be initiated by an inducible control mechanism responsive to a particular stimulatory influence which may be applied to the tumour cells concerned, or which may arise as an endogenous product of the tumour cells, whereby said sensitizing gene or genes are caused or enabled to generate a product that will bring about the death or elimination  
10 of said tumour cells, e.g. through activation of a cytotoxic prodrug or other cytotoxic agent or tumour cell destruction or elimination mechanism.

Thus, from one aspect the invention provides vector material characterised in that contains:

- 15 (a) a tumour cell sensitizing gene or genes of which expression in a tumour cell yields a sensitizing gene expression product having a potential to cause tumour cells to be killed and destroyed, or to be eliminated, or otherwise to be inactivated, or to be rendered sensitive and/or vulnerable to destruction;
- 20 (b) a sensitizing gene expression regulatory system, including promoter means, for said sensitizing gene or genes;
- (c) at least one control gene; and
- 25 (d) a control gene expression regulatory system responsive in use in a transfected cell to the effect of a predetermined exogenous or endogenous expression inducing influence so as to induce expression of said control gene to yield an expression product having a capacity to establish an operative linkage between said sensitizing gene expression regulatory system and said sensitizing



gene or genes effective to trigger and switch on or permit continuous or permanent expression of the latter to bring about continuous production of said sensitizing gene expression product.

The term "gene" is used herein to denote one or more nucleotide sequences, with or without intervening introns, that encodes a functional protein or RNA molecule. It may therefore embrace cDNA sequences. Use of this term in the singular, and of other terms such as gene expression regulatory system, may also cover the plural, and vice versa, where the context so admits. Also, the term "vector" is used herein to denote an agent or vehicle adapted to act as a carrier of nucleic acid fragments or nucleotide sequences inserted therein for the purpose of introducing such fragments or sequences into a prokaryotic (bacterial) or eukaryotic cell. As such, the term "vector" as used herein embraces viruses, including phages, and nucleic acid gene portions thereof, as well as bacterial and synthetic plasmids. Where the vector contains an inserted gene or genes and a regulatory system or promoter which facilitates efficient transcription and, where appropriate, translation of said inserted gene(s), it is termed an "expression vector".

The vector material of this invention is particularly suitable for use in antitumour therapy, in which case it will be introduced into tumour cells, and the following description will generally assume such use although this is not necessarily to be construed as a limitation to the scope of the invention.

In at least some cases where the control gene expression inducing agent is an exogenous agent, such as for example heat or ionizing radiation, it may be sufficient for this to be applied at sub-lethal doses, and/or at sub-therapeutic tumour imaging doses in the case of ionising radiation for example.

The invention may also be defined in one aspect as consisting of expression vector material for use in introducing into tumour cells in the course of antitumour therapy, characterised in that the said vector material contains a

tumour cell sensitizing gene (or genes) and also a gene expression control system adapted to be activated by a control gene expression inducing influence, such as for example ionizing radiation, a chemical inducing agent, non-ionizing electromagnetic radiation or diathermic heat, in such a way as to bring about or  
5 permit permanent or continuous expression in transfected tumour cells of said tumour cell sensitizing gene(s), yielding an expression product that causes or enables said cells to be killed and destroyed, or to be eliminated, or otherwise inactivated, or to be rendered sensitive and/or vulnerable to destruction.

In general, the term "tumour cell sensitizing gene" is used herein to  
10 denote a gene or any DNA sequence or combination thereof which when expressed *in vivo* in a tumour cell generates a product that is effective at least potentially in bringing about the destruction or elimination of such tumour cell and possibly other tumour cells in the vicinity or in metastases.

Various kinds of tumour cell sensitizing genes may be used to yield an  
15 expression product that will give the desired result. In practice, it will often be preferred to use a tumour cell sensitizing gene (or combination of genes) which yields an expression product that is itself a cytotoxic agent or, often more preferably, that is an enzyme or other bioactive agent able to bring about the breakdown or conversion of an inactive prodrug into an active cytotoxic form  
20 or otherwise promotes a cytotoxic effect of another potentially toxic agent. For example, the *Herpes simplex* virus thymidine kinase gene may be used to produce, when transcribed and translated, the thymidine kinase enzyme which is a prodrug activating agent able to convert the inactive prodrug gancyclovir into a cytotoxic metabolite. An effective amount of such prodrug may be  
25 administered systemically, at the same time as transfected tumour cells are subjected to the appropriate control gene expression inducing influence, e.g. ionizing radiation, chemical agent or heat treatment, or before this treatment, or subsequent to this treatment. In another example a sensitizing gene may be

used which expresses an iodine transport protein which can have the effect of causing radioactive iodine, separately administered, to concentrate in target tumour cells such that the latter are killed.

Instead of or as well as providing tumour sensitizing genes or cDNAs  
5 that encode prodrug activating enzymes or other toxic agents, e.g. toxic proteins such as ricin, additional or alternative possibilities include (a) providing DNA sequences or cDNAs that encode immune response stimulating factors intended to bring about the elimination of not only the primary tumour cells but also other tumour cells in tumour metastases, (b) providing DNA sequences  
10 encoding ribozymes, oligoribonucleotides or RNA molecules, especially antisense molecules, that will attenuate the expression of vital proteins or RNA molecules, i.e. any such molecules that are essential for cell survival and propagation, and (c), providing any other cell killing or cell removal mechanisms that can be activated by the expression product of a sensitizing  
15 gene. Hereinafter, these various DNA sequences will be collectively referred to as "genes", and insofar as they may be administered to a mammal for therapeutic purposes they may all be regarded as covered by the term "therapeutic DNA".

Examples of immune response or cytokine genes that stimulate an  
20 immune response in the host in respect of tumour cells include genes that code for GM-CSF, IFN-alpha, IFN-beta, IFN-gamma, IL-1beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-15 and TNFalpha. Other cell killing or sensitizing genes that may be used include Angiostatic genes such as angiostatin, endostatin, IP-10, Mig, PEX, Kringle-5, SDF-1alpha, TIMP-1, TIMP-2, TIMP-3 and TIMP-4,  
25 apoptotic genes such as Bak, Bax, Bcl-XL, Bcl-XS, Bik, SARP-2 and TRAIL; cytolytic genes such as granzymeA, granzymeB and perforin; and gap junction genes such as Connexin26, Connexin32 and Connexin43.

The following points may be noted:

1. Angiostatic genes inhibit the formation of new blood vessels (caused via hypoxia) and would thus increase the level of hypoxia in the tumours, as well as killing (starving to death) cells in the region: (another bystander effect).
- 5 2. Apoptotic genes simply kill cells via apoptosis.
3. Cytolytic genes simply lyse cells.
4. Gap junction genes encode proteins (connexins) that are necessary for the bystander effect as mediated by activated prodrugs (see for example Elshami, A.A., *et al.* (1996) "Gap junctions play a role in the  
10 'bystander effect' of the herpes simplex virus thymidine kinase/ganciclovir system in vitro", *Gene Ther.* 3, 85-92), so co-expression could enhance the effect.

In relation to the bystander effect, it may also be noted that bystander cell killing effects can be observed in tumours that have no direct contact with a  
15 transduced tumour cell that expresses a prodrug activating gene ("distant" bystander effect; Wilson et al 1996; Kianmanesh et al 1997). This appears to be a consequence of an immune response mechanism (Caruso et al 1993; Kianmanesh et al 1997; Misawa et al 1997).

Although it is feasible to arrange for expression of tumour cell  
20 sensitizing gene(s) to be directly under the control of an ionizing radiation or heat responsive promoter or enhancer or other inducible expression-regulatory element of the sensitizing gene expression regulatory system, a potentially serious practical problem results from the fact that such genes presently known which respond to give the highest levels of such inducing influences do so only  
25 transiently. This suggests that in order to achieve effective treatment it would be necessary to apply continuous or repetitive exposure to heat or ionizing radiation or other expression inducing influence. Also, in the case in which

prodrugs are employed, it would be necessary to apply such prodrugs at a particular time for appropriate levels of prodrug activation to occur. Whilst continuous exposure, for example, to ionizing radiation at low doses sufficient for activating radiation responsive promoter elements may be achieved using a  
5 suitable radioactive isotope-labelled tumour specific antibody or ligand, prima facie it would appear that any antitumour treatment methods based for example on exploiting the response of radiation responsive or heat responsive promoter elements to ionizing radiation or to heat or other exogenous expression inducing influence in a gene therapy scenario would necessarily be of limited  
10 value. However, in embodiments of the present invention herein presented there is provided vector material so constructed that expression of the tumour cell sensitizing gene(s) is indirectly under the control of an expression inducing agent responsive element or elements of a control gene expression regulatory system, such element or elements being arranged to have the effect when  
15 activated, albeit transiently, of triggering continuous expression of the tumour sensitizing gene(s), or at least of permitting or enabling continuous expression thereof or priming the cell transfected with such vector material for continuous expression of said sensitizing gene(s).

Continuous expression is achieved in accordance with the invention by  
20 constructing the vector material so that activation of the control gene expression regulatory system induces expression of the control gene in the vector material which encodes an enzyme that acts as a trigger to modify the vector material. for example through a site-specific recombination system, in such a way as to switch on continuous expression of the tumour-sensitizing  
25 gene(s), provided of course that the expression regulatory system of the tumour sensitizing gene(s) is also active. It will of course be appreciated that in some cases the recombination or other vector modification brought about by the expression product of the control gene may not necessarily result in immediate

continuous or permanent expression of the sensitizing gene(s) since the latter may also be conditional on the addition or presence of some other agent, e.g. a chemical agent such as a hormone, or a tetracycline or IPTG in a prokaryotic-based regulation system. In such cases the effect of the activation of the control  
5 gene may be regarded as priming the tumour sensitizing gene(s), permitting expression of the latter.

Regarding the reference to prokaryotic-based expression regulation systems, such as for example the "Tet-On Tet-Off" regulation system and the isopropyl beta-D-thiogalactoside (IPTG) regulation system (see Gossen M,  
10 Bonin AL, Freundlieb S, Bujard H "Inducible gene expression systems for higher eukaryotic cells", *Curr Opin Biotechnol* 1994 Oct;5(5):516-20 ), Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H "Transcriptional activation by tetracyclines in mammalian cells". *Science* 1995 Jun 23;268(5218):1766-9 and Wyborski DL, Short JM "Analysis of inducers of the  
15 E.coli lac repressor system in mammalian cells and whole animals", *Nucleic Acids Res* 1991 Sep 11;19(17):4647-53), these are commercially available (e.g. Clontec and Stratagene) and could also be incorporated in the control gene expression regulatory system.

In some embodiments where antitumour therapy is involved the  
20 expression inducing influence to which the control gene expression regulatory system of the vector material responds is endogenous and tumour related, being produced by tumour cells associated specifically with tumours to which said antitumour therapy is directed. In this case, at least one element of the control gene expression regulatory system may be selected so that the control gene is  
25 automatically upregulated to an effective operational level when the vector material is introduced into cells of the tumours concerned. For example, in some embodiments the control gene expression regulatory system may respond in use in a transfected cell to an expression inducing influence provided by a

change in local oxygen concentration or, alternatively, by a change in environmental thermal conditions in cells containing the vector material, e.g. hyperthermia or hypothermia. To achieve high efficiency for a particular tumour type the selection of at least one element of the control gene expression regulatory system is conveniently carried out using gene array technology (see for example Schena-M; Shalon-D; Heller-R; Chai-A; Brown-PO; Davis-RW "Parallel human genome analysis: microarray-based expression monitoring of 1000 genes", *Proc-Natl-Acad-Sci-U-S-A*. 1996 Oct 1; 93(20): 10614-9, DeRisi-J; Penland-L; Brown-PO; Bittner-ML; Meltzer-PS; Ray-M; Chen-Y; Su-YA; Trent-JM "Use of a cDNA microarray to analyse gene expression patterns in human cancer Nat-Genet", 1996 Dec; 14(4): 457-60, Duggan-DJ; Bittner-M; Chen-Y; Meltzer-P; Trent-JM "Expression profiling using cDNA microarrays". *Nat-Genet*. 1999 Jan; 21(1 Suppl): 10-4 and Watson-A; Mazumder-A; Stewart-M; Balasubramanian-S "Technology for microarray analysis of gene expression", *Curr-Opin-Biotechnol*. 1998 Dec; 9(6): 609-14). Gene array technology may also be used to select efficient sensitizing gene regulatory elements for specific tumours.

In embodiments in which the expression inducing influence is provided by a change in local oxygen concentration this will generally result from a condition involving a reduction in oxygen (hypoxia) within the tumour tissue. Many tumours normally contain regions of hypoxia, particularly where the blood supply has become inadequate owing to the overall proliferation of the tumour cells, so that the hypoxia is present endogenously. In addition, however, it is also known that an effect of ionizing radiation treatment of human tumours is the transient induction of hypoxia within the tumours. These facts can be exploited in connection with the present invention since the promoters of many genes, e.g. the enolase-1 gene described by G.L. Semenza *et al* in *J. Biol. Chem.* 1996, 271, 32529-32537, contain elements responsive to

hypoxia inducing factor-1 (HIF-1) and expression of such genes has been shown to be induced by hypoxic conditions (see for example Wenger, R.H. and Gassmann, M. (1997) "Oxygen(es) and the hypoxia-inducible factor-1", *Biol Chem.* 378, 609-616. Thus, to take advantage of this all that is necessary is to  
5 incorporate a known hypoxia responsive element in the expression regulatory system or promoter of the control gene or genes in preparing the vector material of the present invention.

It would also be possible to synthesise a promoter that contains both radiation responsive and hypoxia-responsive elements so that Cre or other  
10 recombinase enzyme would be expressed when either of the inducing conditions are fulfilled.

A hypoxia responsive element could also be included in the expression regulatory system or promoter of the tumour cell sensitizing gene or genes to control the expression of the sensitizing gene(s) if the tumour cell concerned  
15 were to become hypoxic following priming of the cell after expression of the control gene and operation of the recombinase or other vector modification mechanism.

In most embodiments the expression inducing influence to which the control gene expression regulatory system responds in use in a transfected cell  
20 is provided by an exogenous expression inducing agent applied to cells into which the vector material is introduced. Preferably, the dose level of such exogenous expression inducing agent needed to trigger and switch on expression of the sensitizing gene or genes in the cells of tissue into which the vector material has been introduced is substantially sub-lethal.

25 The exogenous control gene expression regulatory system or elements thereof may be selected to respond in use in a transfected cell to an expression inducing agent which will usually be provided by at least one of the following:



electromagnetic radiation, application of heat or cooling, application of a magnetic or electric field, an exogenous chemical inducing agent, radiation in the form of sub-atomic particles.

- 5           Where the expression inducing agent is electromagnetic radiation it may be in the form of ultra-violet or visible light, or ionizing radiation in the form of X-rays or gamma-rays. With regard to non-ionizing electromagnetic radiation, it is known for example that genotoxic stress produced by ultraviolet radiation can bring about expression of certain genes (see for example Liu ZG, 10 Baskaran R, Lea-Chou ET, Wood LD, Chen Y, Karin M, Wang JY "Three distinct signalling responses by murine fibroblasts to genotoxic stress", *Nature* 1996 Nov 21;384(6606):273-6), and visible light can also upregulate some genes such as the gene of heat shock protein 47 (HSP47) which responds to a wavelength of 652nm, the same wavelength as is used in some photodynamic 15 therapy (PDT) applications (see for example Nagata K Hsp47: "a collagen-specific molecular chaperone", *Trends Biochem Sci* 1996 Jan;21(1):22-6, Verrico AK, Moore JV "Expression of the collagen-related heat shock protein HSP47 in fibroblasts treated with hyperthermia or photodynamic therapy", *Br J Cancer* 1997;76(6):719-24 and Gomer CJ, Ryter SW, Ferrario A, Rucker N, 20 Wong S, Fisher AM "Photodynamic therapy-mediated oxidative stress can induce expression of heat shock proteins", *Cancer Res* 1996 May 15;56(10):2355-60). Radiation in the form of sub-atomic particles may be provided by radioactive isotopes. The effect of magnetic fields in inducing gene expression or upregulation has been reported by Goodman R, "Blank M 25 Magnetic field stress induces expression of hsp70", *Cell Stress Chaperones* 1998 Jun;3(2):79-88.

In some embodiments ionizing radiation in the form of  $\alpha$ -particles may be generated *in situ* in tumour cells transfected with the vector material of this

invention by arranging for the cells to take up a boron compound and then irradiating with low energy neutrons. Since boron compounds are preferentially retained in certain tumour tissue, e.g. in the brain, this so-called boron neutron capture therapy (BNCT) technique (explained more fully in U.S. Patent No. 5599796 of Schinazi *et al.* of which the content is incorporated herein by reference) can provide a useful method of achieving selective antitumour treatment.

Where the expression inducing agent is an exogenous chemical inducing agent it may be a chemical agent that produces cellular damage, e.g. DNA damage or cell membrane damage or other oxidative damage. Such exogenous chemical inducing agent may in the form of an antitumour drug, e.g. a platinum containing drug such as cis-diaminedichloroplatinum (CDDP), commonly known as cisplatin. In this respect it has in fact been found that many expression regulatory elements that are responsive to ionizing radiation are also sensitive to platinum-containing antitumour drugs.

In some cases the expression inducing agent may be an exogenous chemical inducing agent in the form of a hormone that interacts with a receptor molecule which interacts with a complementary hormone responsive element in the control gene expression regulatory system. For examples of hormone control of the expression of specific genes via the interaction of the hormone with a hormone receptor and its subsequent binding, along with accessory factors to the promote regions of hormone responsive genes, see Ferlini C, *et al.* "Tamoxifen induces oxidative stress and apoptosis in oestrogen receptor-negative human cancer cell lines", *Br J Cancer* 1999 Jan;79(2):257-63 and Pratt MA, Satkunaratanam A, Novosad DM "Estrogen activates raf-1 kinase and induces expression of Egr-1 in MCF-7 breast cancer cells", *Mol Cell Biochem* 1998 Dec;189(1-2):119-25.

In practice there is likely to be a wide range of chemotherapeutic

agents or drugs that can be used in combination with appropriate promoter elements as expression inducing agents, especially drugs that have the effect of directly, or indirectly through cellular damage and/or signal transduction pathways, upregulating the expression of endogenous or exogenous genes.

- 5 Potential expression inducing drugs include those that directly or indirectly induce DNA damage such as the alkylating agents. These include methylating agents such as Temozolomide, Dacarbazine, Streptozotocin, and Procarbazine, the nitrosoureas such as Carmustine, Semustine, Lomustine, and Fotemustine, the alkyl sulphonates such as Busulphan and Treosulphan, the nitrogen
- 10 mustards such as Mechlorethamine, Cyclophosphamide, Iphosphamide, Chlorambucil and Melphalan, the ethyleneimines such as triethylene melamine, hexamethylmelamine, TEPA and thio-TEPA, the epoxides such as dibromomannitol and dibromodulcitol, the antimetabolites such as hydroxyurea, Methotrexate, azaserine, Azathioprine, 5-azacytidine, 5-
- 15 fluorouracil, cytosine arabinoside, 6-mercaptopurine, Allopurinol, 6-thioguanine, deoxycoformycin, Tiazofurin, Acivicin, Pyrazofurin and p-aminolaevulinic acid, plant alkaloids such as Vinblastine, Vincristine and Vindesine, Etoposide and Teniposide, antitumour antibiotics such as Doxorubicin, Daunorubicin, Actinomycin, Bleomycins, Mytomycin,
- 20 Mythramycin, Mitozantrone, hormones such as oestrogen and progesterone, and analogues of these agents. In some circumstances, even aspirin may act as a chemical inducing agent, as reported by Fawcett TW, Xu Q, Holbrook NJ "Potentiation of heat stress-induced hsp70 expression in vivo by aspirin", *Cell Stress Chaperones* 1997 Jun;2(2):104-9.

- 25 It will accordingly be appreciated that the control gene expression regulatory system (and possibly also the tumour sensitizing gene expression regulatory system) can comprise a gene upregulation system that can be activated by a wide range of various chemical agents.

It can be noted that some further information about prodrug activating genes (including fusion genes) and also a list of immune response modifying genes is to be found, *inter alia*, on a web site [www.invivgen.com](http://www.invivgen.com) which is to be regarded as part of this disclosure.

5 To provide tumour targeting the inducing agents used may be incorporated in liposomes or may be conjugated to other tumour targeting molecules.

In preferred embodiments the control gene encodes a recombinase enzyme that acts on recombinase target sites to modify the vector material to  
10 establish the required operative linkage between the sensitizing gene expression regulatory system and the sensitizing gene or genes. Preferably the control gene and the recombinase target sites are part of a Cre-loxP or a FLP-FRT site specific recombination system.

In these embodiments, the recombinase target sites are separated by a  
15 region containing a "stop" sequence of nucleotides that blocks or prevents expression of the sensitizing gene or genes until removed by the action of said recombinase enzyme.

Thus, in some preferred embodiments the vector material has an inducible promoter system, including for example an ionizing radiation or heat  
20 responsive promoter element or elements, operatively linked to a control gene that encodes a recombinase enzyme that is able to bring about the recombination of individual short segments of specific DNA sequences in another region of the same vector, or in a separate cotransfected vector, which in turn brings about a resultant activation and expression, or priming for  
25 expression, of the tumour cell killing or sensitizing gene(s). Such specific DNA sequences constitute recombinase target sites and preferably they flank a region called a "Stop cassette" (available for example in Gibco™ plasmid pBS302), which contains transcription termination or stop sequences or which

contain some other intervening sequence that prevents expression of the downstream sequences (n.b. Gibco and Gibco BRL are trade marks of Gibco Europe Limited and/or Life Technologies, Inc.). Such "Stop cassette" is located upstream of the tumour sensitizing gene(s) but downstream of a separate  
5 promoter sequence or sensitizing gene expression regulatory system whereby expression of this tumour sensitizing gene is normally prevented. In these embodiments the vector is constructed so that the recombination of the DNA sequences of the recombinase target sites results in the elimination or deletion of this Stop cassette which is excised from the vector so that transcription and  
10 expression of the tumour killing or sensitizing gene(s) is then no longer prevented.

It will be understood that the control gene expression regulatory system, i.e. the promoter or enhancer element or elements thereof, will generally be such that exposure to the appropriate expression inducing agent,  
15 e.g. heat or ionizing radiation, elicits a response that brings about a substantial, or at least effective, increase in activity and hence transcription and translation of downstream sequences. Preferably the expression control or regulatory system is such that this response can be brought about by very low, non-therapeutic, sublethal doses of the expression inducing agent. Following  
20 activation by such treatment, the promoter or other responsive element(s) will then cause expression of the gene encoding the recombinase enzyme that brings about the sequence-specific recombination of the recombinase target sites which are located in another region of the same vector or in another vector cotransfected therewith, these target sites flanking the region referred to as the  
25 "Stop cassette".

Suitable recombinase genes that may be used in this arrangement include the *Escherichia coli* P1 Bacteriophage *cre* and the *Saccharomyces*

*cerevisiae flp* recombinase genes. Other genes with similar characteristics could also be used.

It should be pointed out that as the gene expression regulatory or control system in eukaryotic cells may comprise a relatively complex promoter consisting of a number of different, non-contiguous, separate parts, and may also include a more remotely located enhancer sequence associated with the promoter, the term promoter element or elements used herein is to be construed broadly as denoting any appropriate part of the expression regulatory or control system.

The *cre* gene, which expresses the Cre recombinase protein of bacteriophage P1, is used in conjunction with *loxP* target or recombination sites. The Cre-*loxP* site-specific recombination system is a well known recombination system (see for example Martina Anton. *et al.*, (1995), "Site-Specific Recombination Mediated by an Adenovirus Vector Expressing the Cre Recombinase Protein: a Molecular Switch for Control of Gene Expression", *Journal of Virology*, 69, 4600-4606, and Minmin Qin. *et al.*, (1995), "Site-specific cleavage of chromosomes in vitro through Cre-Lox recombination", *Nucleic Acids Research*, 23, 1923-1927), and the elements thereof, including the *cre* gene, *loxP* sites and Stop cassette assembled in plasmid vectors, are commercially available, e.g. as a Gibco BRL™ product from Life Technologies, Inc. (U.S.A.). The Flp site specific recombinase system in conjunction with FRT target sites that provide substrates for the Flp recombinase protein (see for example Dymecki (1996) "Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice", *Proc. Natl. Acad. Sci. U.S.A.*, 93, 6191-6196; also U.S. Patents 5654182 and 5677177 of Wahl *et al.*) may be applied in a similar way to excise a blocking Stop cassette so as to "switch on" expression of the tumour

sensitizing gene after activation of the *flp* recombinase gene which is arranged to be under the control of a radiation or heat responsive promoter in the vector.

Examples of promoters or expression control elements that can be activated by low doses of ionizing radiation include the enhancers and/or  
5 promoters or expression control radiation responsive elements of the *egr-1* gene,  $\text{TNF}\alpha$  gene, the *Nfk $\beta$*  gene, the *c-fos* gene, the *jun-b* gene, the *c-jun* and the *c-myc* gene. This list, however, is not exhaustive. A typical example of a heat responsive promoter that may be used is the *hsp-90* gene.

As indicated above, transcription and translation of the tumour  
10 sensitizing gene(s) in the vector generally will be under the control of a separate sensitizing gene promoter or expression regulatory system. This is or includes preferably a promoter that operates very effectively in human cells and most preferably one that will operate specifically in the type of cells that make up the tumour to be treated, by virtue for example of being tissue or cell-type  
15 specific or being associated with a tissue specific enhancer region, or even more preferably one that operates only in tumour cells. Conveniently, this promoter or regulatory system for activating the tumour sensitizing gene(s) may also be selected for good efficiency by the use of gene array technology as previously mentioned and should be located in the vector in such a position that  
20 it does not bring about the expression of any gene at the protein level until a recombination of the DNA sequences of the recombinase target sites occurs. Thus, this sensitizing gene promoter or regulatory system may be located upstream of the Stop cassette which, as pointed out above, is itself located upstream of the tumour-sensitizing gene(s) so as to block or prevent expression  
25 of the latter. Alternatively, this sensitizing gene promoter or regulatory system may be located within a region excised by the action of the recombinase enzyme such that upon recombination it is reorientated so as to be able to

promote continuous transcription and translation of said tumour-sensitizing gene(s) co-excised therewith.

In some embodiments the region between the recombinase target sites contains a duplicate copy of the recombinase control gene together with an associated promoter, this being one example of possible embodiments in which there is more than one control gene system allowing, for example, permanent expression of a recombinase gene or genes after the initial activation by the primary inducing agent.

Examples of mammalian cell promoters for the tumour sensitizing gene(s) that will generally be suitable include the human cytomegalovirus (CMV) gene promoter and the chicken B-actin gene promoter, whilst one example of a tissue specific promoter (in this case appropriate for treating prostatic tumours) is the prostate specific antigen promoter and its associated enhancer region (see for example E. R. Schuur, *et al.*, (1996), "Prostate-specific Antigen Expression Is Regulated by an Upstream Enhancer", *The Journal of Biological Chemistry*, 271, 7043-7051). Examples of tumour-specific promoters, in these cases acting in tumour cells that have lost p53 function, include the HSP-70 promoter (Tsutsumi-Ishii *et al.*, (1995) *Cell growth and differentiation*, 6, 1-8) and the MDR-1 promoter (Zastawy, R.L. (1993) *Oncogene* 8, 1529-1535). For tumour cells that have lost RB-1 function the E2F-1 promoter would be appropriate (M.J. Parr *et al.*, (1997) (see "Tumour-selective transgene expression in vivo mediated by an E2F-responsive adenoviral vector", *Nature Medicine*, 3, 1145-1149).

It will be understood that the sensitizing gene expression regulatory system may incorporate at least one expression inducible element responsive to the effect of a predetermined exogenous or endogenous expression inducing influence in a manner similar to the control gene expression control regulatory system.



It will of course be appreciated that although the control gene expression product will generally act to establish an operative linkage between the sensitizing gene expression regulatory system and the sensitizing gene or genes so as to permit expression of the latter, whether or not expression occurs or continues permanently may depend on the presence of another agent needed to activate the promoter or other expression regulatory element of said sensitizing gene(s). Thus, in the absence of such other agent the action of the control gene expression product may be in effect to switch the sensitizing gene(s) into a "primed" mode ready to yield the active expression product when the sensitizing gene expression regulatory system or promoter is activated. Statements in the present specification should be construed accordingly.

The vector material will generally be composed of vectors constructed in a known manner to provide, as necessary for optimal effect, a polyadenylation signal encoding region at the downstream ends of the recombinase and tumour sensitizing gene(s), and appropriate eukaryotic cell origins of replication. For amplification in bacteria, there will also be included a bacterial origin of replication and an antibiotic resistance gene, as understood by those versed in the art.

As already mentioned, preferred tumour-sensitizing gene(s) include genes that encode prodrug activating enzymes, but other examples are genes that encode cytotoxic proteins or toxins, immune response stimulating factors, ribozymes or antisense RNA molecules. Apart from the *Herpes simplex* virus (HSV) thymidine kinase (*tk*) gene previously mentioned, further examples of tumour sensitizing prodrug activating genes that can be suitable include the *E. coli* nitroreductase (*nr*) gene (see S. M. Bailey *et al.*, (1996) "Investigation of alternative prodrugs for use with *E. coli* nitroreductase in 'suicide gene' approaches to cancer therapy" *Gene Therapy*, 12, 1143/1150), the cytosine deaminase gene, and the mammalian cytochrome p450 2E1 or 2DVI genes.

Other genes that encode proteins involved in different cell killing mechanisms can also be useful.

In a further development, the control gene is a fusion gene that when expressed also produces a fusion protein consisting of an intercellular trafficking protein (such as for example the virion protein VP22 – see for  
5 example Phelan A, Elliott G, O'Hare P "Intercellular delivery of functional p53 by the herpesvirus protein VP22", *Nat Biotechnol* 1998 May;16(5):440-3 and Elliott G, O'Hare P Intercellular trafficking and protein delivery by a herpesvirus structural protein, *Cell* 1997 Jan 24;88(2):223-33) and a  
10 recombinase protein. Also, the tumour cell sensitizing gene or genes may comprise a fusion gene sequence that when expressed produces an intercellular trafficking protein and a tumour cell sensitizing protein.

In some cases, the tumour cell sensitizing gene or genes and the control gene are in separate vectors, but in other cases the tumour cell  
15 sensitizing gene or genes and the control gene are in the same vector.

If desired, it is also possible to include in the vector material a number of different control gene expression regulatory elements responsive to different expression inducing influences so as to be activated under a range of different conditions.

20 Also, in some embodiments a plurality of tumour sensitizing genes will be incorporated which may provide a range of different expression products for killing or eliminating the tumour cells. This may be useful, for example, where the sensitizing genes are intended to express prodrug activating agents – by  
25 arranging for a set of sensitizing genes to produce a range of different prodrug activating agents the most efficient prodrug may be selected for systemic administration in each particular case of patient treatment.

It is also contemplated that the vector material of this invention may be presented as a kit which may comprise one or more unit doses of the vector material, herein defined, together with a transfection agent. In such kits the control gene(s) and sensitizing gene(s) may be contained either in the same  
5 vectors or in separate vectors, and where the sensitizing gene(s) are designed to express prodrug activating agents for use in antitumour therapy, the kits may also include pre-prepared doses of a pre-selected prodrug or a range or prodrugs to be selected at the time of administration. The kits may also contain a range of different transfection agents that also may be selected at the time of  
10 administration.

The provision of such kits constitutes a further aspect of the invention.

The vector for delivery of the therapeutic DNA to patients can be a retroviral, lentiviral, adenoviral, adenovirus-associated viral, or an Epstein-Barr viral based vector or any viral or bacterial vector delivery system that might be  
15 used for gene therapy in humans. Alternatively, it can be a non-viral vector that would be made up for administration in a suitable formulation such as, for example, a complex with cationic liposomes or with a tumour-targeting antibody or ligand, or that would be incorporated into some other non-viral DNA delivery system for delivery to tumour tissue, especially human tumour  
20 tissue. In general, the vector will have the ability to invade tumour cells and to express the encoded tumour sensitizing genes(s) therein following exposure of transfected cells to the appropriate inducing agent. Delivery of the vector material will usually be carried out according to generally accepted gene therapy procedures or methods as described, for example, by Friedman in  
25 *Therapy for Genetic Disease*, T. Friedman, ed., Oxford University Press (1991) and reviewed by I.M.Verma and N. Somia (1997) in "Gene therapy-promises, problems and prospects" *Nature* 389, 239-242. For example, after constructing the viral or plasmid vector or vectors containing the tumour cell sensitizing

gene(s) and expression control element(s) described, this vector material may be incorporated in a pharmaceutical composition, possibly in combination with a pharmaceutically acceptable excipient or carrier vehicle and a transfection agent, for example a transmembrane carrier such as Penetratin™. This composition may then be injected into the patient, either locally at the site of the tumour or systemically. Such pharmaceutical compositions or formulations represent a further aspect of the invention.

As indicated above, gene transfer methods known in the art which may be useful in the practical application of the present invention include both viral and non-viral transfer methods. Viruses that have been used as gene transfer vectors include for example papovaviruses, vaccinia viruses, herpesviruses, as well as adenoviruses, lentiviruses, adeno-associated viruses and retroviruses of avian, murine and human origin. Many human gene therapy protocols have been based on disabled murine retroviruses.

Non-viral DNA transfer methods known in the art include mechanical techniques such as micro-injection, membrane fusion-mediated transfer via liposomes (as already mentioned), and direct DNA uptake and receptor-mediated DNA transfer. Also, viral-mediated DNA transfer can be combined with direct *in vivo* DNA transfer using liposome delivery which may allow one to direct the viral vectors to the tumour cells concerned rather than into the surrounding normal cells. Alternatively, one may inject into tumours a self-replicating retroviral vector producer cell line so that there could then be a continuous source of DNA vector particles, similar to a technique already approved for use in humans with inoperable brain tumours. Although the vectors may be taken up directly by cells, actively or by diffusion, liposome mediated transfer may be best achieved in some cases by use of a transfection agent such as a cationic lipid, e.g. the compound N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium methylsulfate, commonly known as

DOTAP, marketed by Boehringer Mannheim. However, many other suitable methods will be known to persons familiar with gene therapy techniques. An important aspect of many embodiments of the invention is that the vector delivery system may not need to have absolute specificity for tumour cells since  
5 the tumour cell killing mechanism will only be activated in the area affected by the expression inducing agent which may be accurately targeted, e.g. as with radiation or diathermy, and in some embodiments the killing mechanism may only operate in cancer cells by virtue of tumour-specific promoters driving the expression of the tumour-sensitizing gene(s).

10 For clinical use, the vectors may be mixed with a selected transfection agent to provide a pharmaceutical preparation which may be administered by any suitable means, for example parenterally, orally or perhaps topically. In at least some cases such pharmaceutical preparations will be in the form of a sterile liquid formulation, presented possibly in unit dosage form in sealed  
15 ampoules ready for use, and as already mentioned delivery or administration may be effected by injection, e.g. directly into tumour tissue or intravenously. In practice, not only the method of administration but also the particular protocol employed may be important; however, the precise details of the treatment and appropriate dosages will generally be determined by carrying out  
20 straightforward trials and by the general experience of the medical practitioners in charge of the treatment.

The invention also extends to methods for treating tumour cells in a biological host system which, at least in one embodiment, comprises:

- 25 (a) administering to the system an effective amount of a composition comprising vector material containing a tumour cell sensitizing gene or genes and having a control gene expression regulatory system responsive to a predetermined exogenous or endogenous expression inducing influence, said control gene expression regulatory system

being operatively linked to a recombinase gene, together with recombinase target sites flanking a region of which removal permits continuous expression of said tumour cell sensitizing gene(s) as specified above, or primes said sensitizing gene(s) for continuous  
5 expression;

(b) causing said tumour cells transfected with said vector material to be subjected to a dose of an expression inducing agent effective to activate the recombinase gene expression control system of said vector material, thereby to bring about, via recombinase-mediated site  
10 specific recombination within the vector material. expression of the or each tumour cell sensitizing gene component;

and, in the case of prodrug activating genes,

(c) administering to the host an effective amount of a composition comprising a prodrug convertible into an active form by the expression  
15 product of said tumour cell sensitizing gene or genes.

Usually, in most embodiments, the dose of the expression inducing agent, e.g. diathermy heat or ionizing radiation, will be derived from a directed external source or from a radioisotopically labelled tumour-seeking or cell or tissue-type seeking agent. Examples of suitable radioisotopically labelled  
20 tumour-seeking agents for use in the above method include materials such as metaiodobenzylguanidine (MIBG) or suitably radioisotopically labelled tumour cell specific antibodies or ligands.

The prodrug referred to above may for example comprise any of the following compounds, administered either singly or in appropriate  
25 combinations: gancyclovir, CB1954, fluorouracil, dacarbazine, or cyclophosphamide, subject to the prodrug activating gene(s) being, respectively, a gene or genes or cDNAs encoding HSV thymidine kinase,

nitroreductase, cytosine deaminase, cytochrome p450 2E1 or cytochrome p450 2DVI.

To summarise, in general in preferred embodiments:

- 5 (1) where an ionizing radiation or platinum antitumour drug responsive expression control system is used, the responsive element or elements is or are selected from the radiation responsive enhancer or promoter of the *egr-1* gene, the *TNF $\alpha$*  gene, the *Nfk $\beta$*  gene, the *c-fos* gene, the *jun-b* gene, the *c-jun* the *c-myc* gene and their functional equivalents, and either a single such radiation responsive element may be used or a  
10 plurality of tandemly arranged radiation-responsive DNA sequence elements (all the same or different) may be used in an array thereof;
- (2) where a heat-responsive promoter or expression control element is used, this is the promoter of the *hsp-90* gene or its functional equivalent;
- 15 (3) the recombinase gene contained in the vector material will be the *E.coli* bacteriophage P1 *cre* gene or the *Saccharomyces cerevisiae* *FLP* gene, or a functional equivalent of one or other of these genes;
- (4) the DNA sequence of the vector material will include polyadenylation signal encoding regions from the virus SV40 or equivalent inserted at  
20 appropriate locations;
- (5) the vector material may contain a mammalian cell promoter which is that of the cytomegalovirus (CMV) or that of the chicken B-actin gene, or a functional equivalent of these;
- 25 (6) the vector material may contain a tissue specific promoter (e.g. the prostate specific antigen promoter and its associated enhancer region, or an equivalent of this) that is specific for the particular tumour type to be treated;

- (7) the vector material may contain a tumour specific promoter such as the HSP-70 promoter, the MDR-1 promoter, the E2F-1 promoter or telomerase-related promoters (or their equivalents), according to the p53 or RB-1 or telomerase status of the tumour;
- 5 (8) the DNA vector material will contain recombinase target sites provided by the *loxP* sites (or equivalents) that are substrates for the Cre recombinase enzyme, or recombinase target sites provided by FRT sites (or their equivalents) that are substrates for the Flp recombinase enzyme, or equivalents;
- 10 (9) the vector material will contain a Stop cassette provided by a sequence that prevents expression of any protein coding sequence located downstream thereof unless this region is excised by the action of the recombinase, or by another mechanism that results in a change in orientation or loss of this Stop sequence;
- 15 (10) the tumour sensitizing gene is selected from the *E.coli* nitroreductase gene, cytosine deaminase (CD) gene, *Herpes simplex* virus thymidine kinase (HSV-*tk*), mammalian cytochrome p450 2E1 or 2DVI gene, and their functional equivalents;
- (11) The vector or each vector of the vector material will contain a bacterial  
20 origin of replication;
- (12) The vector or each vector of the vector material will contain at least one mammalian cell origin of replication;
- (13) The vector or each vector of the vector material will contain a bacterial antibiotic resistance gene;
- 25 (14) The vector or each vector of the vector material will contain intron sequences;



- (15) The vector or each vector of the vector material may contain a mammalian cell antibiotic resistance gene.

The invention also includes use of vector material or vectors as  
5 hereinbefore specified in the manufacture of a medicament or of a kit for use in antitumour therapy.

By way of example of the manner in which the invention may be carried out illustrative embodiments and background work in developing the invention will now be described in more detail with reference to the accompanying drawings. The particular embodiments and examples illustrated and described, however, should not be construed in any way as a limitation on the scope of the invention.

### BRIEF DESCRIPTION OF THE DRAWINGS

Schematic diagrams (not to scale) of vectors in accordance with the invention and containing the above-mentioned elements are shown in the accompanying drawings. In these drawings:

FIGURE 1 represents one embodiment of a gene therapy plasmid vector, herein labelled "pComplete1", containing an ionizing radiation responsive recombinase expression control system;

FIGURE 2 shows the structure and some of the components making up pComplete2, a modified version of the vector in Figure 1 (the curved arrows adjacent to the Cre and tk ORFs indicate the direction of Cre and tk transcription following recombination at the loxP sites);

FIGURE 3 shows the structure and some of the components making up pComplete3, another version of the vector illustrated in Figure 1;

FIGURE 4 is a diagram showing different stages in the construction of the pComplete1 vector which contains tandemly arranged radiation-responsive elements of the *egr-1* promoter (double-framed boxes indicate starting materials);

FIGURE 5 is a schematic diagram demonstrating how pComplete1 responds to ionising radiation, undergoes recombination, and permanently expresses the tumour sensitizing gene thymidine kinase resulting in gancyclovir activation and cell killing;

FIGURE 6 is a diagram showing stages in the construction of vectors pEGRL(b)-*cre* and pStop-*gfp* which were introduced into MCF-7 cells and used in testing the principle of the invention;

FIGURE 7 is a bar chart diagram showing FACS analysis of MCF-7  
5 cells following transfection with pStop-*gfp* only (light grey bars) or pStop-*gfp* together with pEGRL(b)-*cre* (dark grey bars) and exposure to 5Gy or 10Gy (in two 5Gy doses) of ionising radiation. Controls were not irradiated (0Gy);

FIGURE 8 is a diagram showing the results of a further test in which  
10 MCF-7 cells were transfected with pEGRL(b)-*cre* and selected using the antibiotic G418, giving rise to the cell clone pCE which was, in turn, transfected with pStop-*tk* and then subjected to radiation. in the absence (open circles) of and in the presence (closed circles) of the prodrug ganciclovir;

FIGURE 9a and 9b are diagrams showing tumour cell growth *in vitro*  
in the presence of the prodrug ganciclovir, after increasing doses of radiation.  
15 Figure 9a relates to MCF-7/E4*cre* cells (○), MCF-7/E4*cre* cells transfected with plasmid p*Stk* (●) and MCF-7 cells transfected with p*tk* (▼). Figure 9b MCF-7 cells (○), MCF-7 cells transfected with pE4*tk* (■). Error bars are shown in one direction only for clarity;

FIGURE 10 is a bar chart diagram illustrating induction of  
20 fluorescence in MCF-7 cells transfected with plasmid vectors pE4GFP and exposed to cisplatin (1 micromolar), ionising radiation (5Gy) or both. Controls were unexposed; and

FIGURE 11 is a bar chart diagram illustrating FACS analysis results  
for induction of fluorescence in NB1G human neuroblastoma cells transfected  
25 respectively with plasmids pEGFP (which contains a hypoxia-responsive promoter) after incubation in the absence or presence of 25 micromolar CoCl<sub>2</sub> in the cell culture medium.

### DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

Referring to the drawings, in the particular example of the plasmid vector pComplete1 depicted in Figure 1 the ionizing radiation responsive recombina-  
5 se expression control system is provided by a promoter sequence comprising a synthetic tandem array of radiation-responsive elements corresponding to radiation-responsive elements found in the promoter of the *egr-1* gene, and is located upstream of the cytomegalovirus immediate-early (CMV I.E.) promoter. A control gene providing a recombina-  
10 se protein coding sequence or open reading frame (ORF), which in this example is that of the P1 bacteriophage *cre* gene (labelled *Cre* ORF), is located downstream of an intron sequence, the latter serving to enhance the translation of the downstream ORF. There is also in this plasmid vector a strong constitutive mammalian cell enhancer/promoter which is that of the cytomegalovirus gene (labelled CMV  
15 I.E. Enhancer/promoter), *loxP* recombina- se target sites that provide substrates for the Cre recombina- se enzyme, and a tumour sensitizing gene which in this example is the Herpes simplex virus thymidine kinase (*tk*) gene (labelled *tk* ORF). As indicated, the *loxP* sites flank a Stop cassette. Downstream of each ORF is located a polyadenylation signal-encoding sequence which in this case  
20 is derived from the Simian virus 40 (SV40) early gene and is labelled SV40 polyA site in the diagram of Figure 1.

The term enhancer/promoter is used herein to denote combined or fused enhancer and promoter nucleotide sequences.

Following recombination, the above-mentioned mammalian cell  
25 enhancer/promoter drives expression of the *tk* gene. Possible alternatives include the promoter of the chicken B-actin gene, a tissue specific promoter such as for example the prostate specific antigen promoter and its associated enhancer region, or a tumour specific promoter such as for example the HSP-70

promoter, the MDR-1 promoter, the E2F-1 promoter or telomerase-related promoters.

As pointed out, other possible tumour sensitizing genes that may be used include the *E.coli* nitroreductase or cytosine deaminase genes, and the  
5 mammalian cytochrome p450 2E1 or 2DVI cDNAs. Possible alternatives for the above-mentioned radiation-responsive promoter for the recombinase gene include promoters comprising different numbers of radiation-responsive elements, which may be the same or different, arranged in tandem, the entire enhancer/promoter DNA sequence, or only the enhancer DNA sequence of the  
10 *egr-1* gene. Other alternatives include the sequences of the *TNF $\alpha$*  gene, the *Nfk $\beta$*  gene, the *c-fos* gene, the *jun-b* gene, the *c-jun* and the *c-myc* gene or their functional equivalents. Where a heat-responsive promoter or expression control element is used, as previously mentioned this can be the promoter of the *hsp-90* gene or its functional equivalent.

15 In Figure 1 the region labelled "Neo gene" indicates the aminoglycoside phosphotransferase gene ORF complete with a mammalian cell promoter and SV40 polyadenylation site, which is incorporated for selective growth of transfected mammalian cells *in vitro* in the selective antibiotic G418. This allows the vector to be used as a model to demonstrate aspects of the  
20 invention *in vitro* in cultured human cells since it allows permanent cell lines to be established. Such cell lines, e.g. the pCE cell line (see later), can be used not only to demonstrate the working of the invention *in vitro* but they can also be used to produce tumour xenografts in immune-deficient mice for demonstrating the working of the invention *in vivo*. Also as shown in Figure 1, the plasmid  
25 contains an antibiotic resistance gene expression cassette such as that conferring resistance to ampicillin in *E.coli*, (labelled Amp in Figure 1), a bacterial origin of replication for growth of the plasmid in *E.coli* (labelled *E.coli* ori in Figure 1), and a mammalian cell origin of replication for

replication of the plasmid in mammalian cells (not shown). The latter would be ideally located outside the region excised by the recombinase so that additional copies of the inactive plasmid might be produced by DNA replication prior to recombination. and following recombination additional copies of the activated  
5 plasmid, now expressing the tumour killing or sensitizing gene(s), might also be produced by DNA replication, resulting in increased expression of the tumour killing or sensitisation gene(s).

The vector illustrated in Figure 2 (pComplete2) contains the same elements as are shown in Figure 1 but the region between the *loxP* sites has  
10 been modified to contain another copy of the recombinase Cre ORF and of the SV40 poly A site, the direction of transcription of these sequences being opposite to that of the *tk* gene. Another copy of the CMV I.E. enhancer/promoter is located adjacent to the Stop cassette and there is another intron located transcriptionally upstream of this. These elements are positioned  
15 such that excision and recombination of the region between the *loxP* sites by Cre (supplied by a radiation-upregulated Cre expression cassette located elsewhere in the plasmid as shown in Figure 2) results in the production of two circular plasmid molecules, the *tk* gene being expressed from one. while the *cre* gene in the other is now able to be expressed as a result of the repositioning of  
20 the CMV I.E. enhancer/promoter upstream of the Cre ORF. This should ensure that any copies of the switchable plasmid which are present in the cells but which have not become activated by the initial removal of the Stop cassette would then be activated, without the requirement for additional doses of radiation or diathermy. Again, the Cre-expressing circularised fragment can be  
25 designed to contain a mammalian cell origin of replication so that additional copies of the activated plasmid might be produced by DNA replication, resulting in increased expression of Cre.

The vector in Figure 3 (pComplete3) is designed so that that excision of the Stop cassette region between the *loxP* recombination sites generates a DNA fragment in which two different tumour sensitizing (or killing) genes flanking an internal ribosome entry site (IRES) come under the influence of a mammalian cell enhancer/promoter or tumour-specific enhancer/promoter also contained within that fragment, giving rise to continuous expression of both these two genes. The example shown in Figure 3 is of the *tk* gene and the E.coli nitroreductase gene (labelled Nitroreductase-ORF) that would be expressed by virtue of the CMV I.E. enhancer/promoter, following Cre-mediated recombination.

It will be appreciated that many variations of the above-described vectors can be produced. For example, in pComplete2 the positions of the Cre ORF and *tk* ORF flanking one of the *loxP* sites can be reversed so that, following the action of Cre, the shorter region between the *loxP* sites will express the tumour sensitizing gene whilst the residual vector will continuously express Cre. Furthermore, additional IRES will allow the expression of more than one protein or other tumour cell killing molecule from both of the vector fragments following recombination and both fragments might contain a variety or mixture of the Cre ORF and many other tumour sensitizing genes, and/or tissue or tumour-specific promoters or enhancer/promoters.

#### Vector Construction

By way of example, the manner of construction of the pComplete1 vector in accordance with the invention will now be described in somewhat greater detail.

The elements of the vector are assembled in standard ways well-known in the art of recombinant DNA technology from components available commercially or that can be made synthetically or derived by PCR-amplification of human DNA, and appropriate restriction endonuclease sites are

introduced which can be used to produce fragments with compatible termini. As hereinafter more fully described, these may then be ligated together as required.

The components of the vector(s) in this specific example are  
5 conveniently obtained as summarised below:

- (a) The or each tandem array of radiation responsive promoter elements corresponding to the radiation responsive promoter element of the *egr-1* gene is conveniently synthesised initially as a short single-stranded oligonucleotide as described by Weichselbaum *et al.* (1994)) "Gene  
10 therapy targetted by radiation preferentially radiosensitizes tumour cells", *Cancer Research*, 54, 4266-4269, the content of this paper being incorporated herein by reference. The terminal regions of this single-stranded oligonucleotide can be designed so that, following annealing to a suitable complementary oligonucleotide, the resulting  
15 double-stranded molecule can be ligated into the plasmid vector pCI-neo (commercially available from Promega) following its digestion with appropriate restriction endonuclease/s, in this case *Bgl*II and *Sgf*I.
- (b) The Cre recombinase protein coding sequence of the control gene is readily obtained as a *Xho*I/*Mlu*I fragment from the commercially  
20 available plasmid pBS185 (Gibco BRL™ - Life Technologies, Inc.).
- (c) The cytomegalovirus (CMV) immediate-early (I.E.) enhancer/promoter, SV40 polyadenylation signal, the *E.coli* and mammalian cell origins of replication, ampicillin resistance gene and aminoglycoside resistance cassette (comprising the CMV I.E.  
25 enhancer/promoter, aminoglycoside phosphotransferase ("neo") protein coding sequence and SV40 polyadenylation signal) are also conveniently obtained from the plasmid vector pCI-neo commercially available from Promega.



- (d) The cytomegalovirus (CMV) immediate-early (I.E.) enhancer/promoter, and a suitable multiple cloning site (MCS) is obtainable as a *Bam*HI/*Bgl*II fragment from plasmid pCI, also commercially available from Promega.
- 5 (e) The *lox*P-flanked Stop cassette can be obtained as an *Eco*RI/*Spe*I fragment from Gibco™ plasmid pBS302.
- (f) The *Herpes Simplex* virus *tk* gene has the nucleotide sequence originally published by Wagner, M.J. *et al.* (1981) "Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type-1", *Proc. Natl. Acad. Sci. USA*, 78, 1441-1445 and for the purpose of
- 10 the present invention it is conveniently cloned by standard methods into plasmid pBR322 (Gibco) to produce a plasmid vector here termed pBR-*tk* which provides a carrier and reservoir of the gene.

More specifically, the production of the vector pComplete1 shown in

15 Figure 1 from synthetic oligonucleotides, plasmids pCI-*neo* and pCI (Promega), plasmids pBS302 and pBS185 (Gibco) and the above-mentioned pBR-*tk* plasmid can be carried out in a series of stages substantially as described below with reference to the scheme illustrated in the diagram of FIGURE 4, and with reference to the oligonucleotide sequences shown in TABLE 1 at the end of the

20 present description.

In the diagram of FIGURE 4, the double-framed boxes indicate starting materials, and the various stages may be carried out substantially as follows:

- (1) The CMV I.E. enhancer fragment is removed from plasmid pCI-*neo*
- 25 following digestion with *Bgl*II and *Sgf*I and is replaced with a double stranded oligonucleotide labelled "RR-elements(a)". This contains a tandem array of 6 radiation-responsive elements and is produced by

annealing synthetic oligonucleotides EGRE1 and EGRE2 (see Table 1) to generate the plasmid, labelled pEGRL(a)-neo.

5 (2) The *cre* recombinase gene protein coding region, obtained as a *XhoI/MluI* fragment of pBS185, is inserted into *XhoI/MluI*-digested pEGRL(a)-neo to generate pEGRL(a)-*cre*.

(3) The *NotI* site in the pEGRL(a)-*cre* Multiple Cloning Site (MCS) is deleted by *NotI* digestion, then the resultant single-stranded termini filled-in using the Klenow enzyme, followed by self-ligation of the plasmid.

10 (4) The CMV I.E. promoter/enhancer along with its associated multiple cloning site is obtained as a *BamHI/BglII* digest fragment from plasmid pCI, and is ligated into the *NotI*-deleted pEGRL(a)-*cre*, following partial digestion of the latter with *BamHI*, thereby to produce pEGRL*cre*MCS2.

15 (5) The Stop cassette, obtained from the Gibco™ plasmid pBS302 by digestion with *EcoRI* and *SpeI*, is ligated into *EcoRI/XbaI* digested pCI-*neo* plasmid to generate pStop.

20 (6) The *tk* gene protein coding region is obtained as a PCR amplification product of pBR-*tk* using the primers *Clark* (SEQ ID No: 9) and *tkNot* (SEQ ID No: 10) shown in TABLE 2 at the end of the present description. Following *ClaI* and *NotI* digestion the PCR product is ligated into *AccI/NotI*-digested pStop to generate pStop-*tk*.

25 (7) The *NotI/EcoRI* fragment of pStop-*tk* containing the Stop cassette and the *tk* ORF is ligated into *EcoRI/NotI*-digested pEGRL*cre*MCS2 to generate pComplete1.

### Radiation Switched Operation

Figure 5 illustrates in relation to pComplete 1 the molecular switching process in which Cre recombinase transcription and translation is induced following exposure to ionising radiation. The Cre enzyme expressed acts upon  
5 pComplete1 to excise the Stop cassette by recombination at the *loxP* sites. This results in the formation of a small circular DNA fragment containing the Stop cassette and a *loxP* site and of a recircularised plasmid (pComplete1: activated form) that transcribes and translates the thymidine kinase DNA sequence. The expression of this enzyme can then in turn cause activation of the prodrug,  
10 gancyclovir (provided by systemic injection) which will kill host cells and also cells in contact with the host cells (via the bystander effect).

### Modified Vectors

Alternative vectors containing different promoter and/or enhancer elements can be constructed by analogous methods. Thus, as indicated earlier,  
15 the vectors may be constructed so as to contain the entire radiation responsive *egr-1* enhancer/promoter. This comprises nucleotides -676 to +10 according to Sakamoto *et al.* ("5' Upstream sequence and genomic structure of the human primary response gene *egr-1*/TIS8" (1991) *Oncogene* 6, 867-871) and can be isolated from human DNA by PCR amplification using the primers EGRE6  
20 (SEQ ID No: 7) and EGRE5 (SEQ. ID NO: 6) shown in TABLE 2. The enhancer region alone (nucleotides -676 to -178) can be isolated from human DNA by PCR amplification using the primers EGRE5 (SEQ. ID NO: 6) and EGRE7 (SEQ. ID NO: 8) shown in TABLE 2. These primers, which are  
25 designed to introduce appropriate restriction endonuclease or other suitable sites for cloning, are conveniently synthesised using commercially available oligonucleotide synthesising apparatus. The human DNA required for carrying out the PCR amplification of these radiation responsive sequences is conveniently isolated from HeLa cells by a conventional method, as described

for example by Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989), "Molecular Cloning - A Laboratory Manual", *Cold Spring Harbor Laboratory Press, U.S.A.*).

Alternative recombinase control genes and target sites as well as other  
5 tumour sensitizing mechanisms can also be used. As previously mentioned, one example of the latter is the *E.coli* nitroreductase gene in respect of which the protein coding sequence (nucleotides 166 to 831) can be isolated by PCR amplification of *E.coli* strain B DNA using oligonucleotide primers designed to introduce suitable restriction endonuclease sites for cloning purposes.

10 In modifications in which the components of the vectors are arranged in different ways in order to achieve the required trigger and switching effect of radiation (or other expression inducing influences), as has been described in relation to the vector illustrated in the diagram of Figure 2 the region between the recombinase (*loxP*) target sites can contain a duplicate copy of the  
15 recombinase gene and another mammalian cell promoter on opposite sides of the Stop cassette, the arrangement being such that the recombination and re-orientation brought about by the action of the recombinase initially expressed will bring this second copy of the recombinase gene under the influence of this other promoter, thereby resulting in the continuous expression of this second  
20 copy of the recombinase coding sequence. As has already been pointed out, continued expression of the recombinase enzyme after triggering by a single dose of radiation will ensure that further radiation or heat treatment will not be required if there were to be inadequate or inefficient recombination of the vectors in the tumour cells due to low levels of heat or radiation-induced  
25 expression of the recombinase after the initiating dose of radiation or heat. In this way the dose of heat or radiation or other expression inducing influence required to trigger the removal of the Stop cassette and the activation of the silenced expression cassette or tumour sensitizing gene(s) in every copy of the

vector taken up by the cells can be kept minimal. This can be particularly important if the system of vector delivery results in the uptake of more than one copy of the vector into the host cells or if the vector undergoes replication before recombinase-mediated recombination.

5           Additionally, multiple killing or sensitisation factors can be expressed in a similar way by means of internal ribosome entry sites (IRES) that allow the expression of more than one protein coding sequence under the influence of a single promoter region, as has been described in connection with the vector of Figure 3. This can allow, for example, the simultaneous expression of different  
10   prodrug activating proteins and immune response stimulating factors, or different combinations thereof, or different combinations of prodrug activating proteins and other proteins, including Cre, or other molecules that kill cancer cells. It is also possible to express fusion proteins having more than one functional prodrug activating activity.

15           The vectors may be designed to be episomal, requiring for example an Epstein-Barr virus nuclear antigen-1 gene element and origin of replication, or a SV40 origin of replication. Alternatively, they may be designed to integrate into host cell DNA as would be the case for retroviral or adenoviral vectors. The excised region between the recombinase target sites or the region outside  
20   the recombinase target sites encompassing the tumour killing or sensitizing gene(s) may also be designed to be episomal and to have its own mammalian cell origin of replication. It is recognised in some cases that the capacity of certain viral genomes to harbour DNA is size-limited and therefore some components of the vectors herein illustrated may need to be eliminated in order  
25   that the essential genetic information can be accommodated.

### Testing

The operation of the system can be tested in cultured human tumour cells and xenografts established therefrom using vectors of the general kind herein described.

5 (a) Demonstration of radiation-mediated switching in human tumour cells

In order first to prove the "molecular switching" principle of the invention, a series of experiments was carried out to test the operation of the various components individually and then in combination. For convenience and for the purposes of accurate quantitation of any effect observed, instead of a  
10 prodrug activating gene, the gene encoding the Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* was used as a sensitizing "reporter" gene. The ORF of this gene was cloned into the vector pStop (see Figure 6) downstream of the Stop cassette to generate pStop-*gfp*. This plasmid, which should not be able to express GFP unless it undergoes recombination, was  
15 transfected into MCF-7 cells in order to provide a control.

In constructing pStop-*gfp*, the Green Fluorescent Protein (GFP) open-reading frame was excised from plasmid pEGFP-1 (obtained from Clontech) with *Cla*I and *Not*I and was cloned into the *Acc*I/*Not*I sites of pStop. The radiation-responsive synthetic *Egr-1* enhancer (E4) formed by annealing  
20 EGRE3 and EGRE4 (see Table 1) containing four repeats of the previously defined decanucleotide radiation-responsive expression control elements (see Datta *et al.*, (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 101149-101153) was cloned into the pCI-*neo* vector to generate pEGRL(b)-*neo*. The *cre* gene was obtained from pBS185 as an *Xho*I/*Mlu*I fragment and was ligated into  
25 *Xho*I/*Mlu*I-digested pEGRL(b)-*neo* to generate pEGRL(b)-*cre*. This, together with the plasmid pStop-*gfp*, was transfected into MCF-7 cell lines using the cationic lipid transfection reagent Lipfectamine. pStop-*gfp*-transfected and pStop-*gfp* plus pEGRL(b)-*cre*-transfected cells were irradiated (5Gy and 10Gy

in two 5Gy doses), and the number of fluorescent cells within a constant aliquot of the transfected population was measured by FACS, as hereinafter described. As can be seen in Figure 7, transfection with the pStop-*gfp* vector alone resulted in very low numbers of fluorescent cells, although there was a slight  
5 increase in this number following irradiation. Transfection with pStop-*gfp* plus pEGRL(b)-*cre* resulted in a larger number of fluorescent cells than with pStop-*gfp*-transfected irradiated cells, but this number was further increased to a much greater extent following irradiation. It can thus be concluded that irradiation activated the radiation-responsive promoter in pEGRL(b)-*cre* which resulted in  
10 the expression of Cre recombinase which in turn recombined the pStop-*gfp* vector, causing expression of GFP. This therefore demonstrated the operation of the general "switch" principle described in connection with this invention.

(b) Demonstration of radiation-mediated killing in human tumour cells

To test this aspect, MCF-7 cells were transfected as described above  
15 with the plasmid pEGRL(b)-*cre* and a permanent cell line, pCE, was established by G418 selection. This was transfected with the plasmid pStop-*tk* described earlier and the cells were plated in 96-well microtitre plates in either complete tissue culture medium (RPMI + 10% foetal calf serum) or the same containing 50 mM ganciclovir (GCV). Eight hours after plating the cells were  
20 irradiated as an attached monolayer ( $^{60}\text{Co}$  gamma rays, approx 1Gy/min) at 37°C. MTT assays were performed 24 hours later and radiation survival data were presented as a mean ( $\pm$  SD) of 48 replicates per dose point.

As shown in Figure 8 there was some degree of cell killing with increasing doses of radiation in the absence of GCV. However, in the presence  
25 of GCV, there was substantially more killing at doses of 2Gy or more. This is consistent with radiation-mediated upregulation of the *cre* recombinase gene in the pCE plasmid and subsequent recombination of the *loxP* sites, resulting in the removal of the stop cassette, leading to the synthesis of thymidine kinase

and subsequent action of the latter on GCV to convert it to a cytotoxic metabolite. The absence of an effect at the low dose of 1Gy is also consistent with this being below a threshold dose for activation of the radiation-responsive promoter in the *cre*-encoding vector. The increased cell killing at 5Gy may reflect synergism between radiation and activated GCV.

(c) Further testing and demonstration of radiation-mediated gene therapy treatment of tumour cells

In another series of experiments, a cell line MCF-7/E4*cre* was established that constitutively contained a radiation-inducible *Cre* recombinase gene. MCF-7 cells were transfected with a plasmid designated pE4*cre* containing a *Cre* gene and the synthetic *Egr-1* enhancer/promoter E4. These cells were then subjected to G418 selection. Cell lines were cloned by serial dilution in G418 (0.5 mg mL<sup>-1</sup>). The same technique was used to establish a cell line designated MCF-7/E4*tk* composed of MCF-7 cells transfected with a *tk* gene containing plasmid pE4*tk*. MCF-7 cells and cell lines were transfected with pE4*cre*, p*Stk* or p*tk* plasmids, p*Stk* being a plasmid containing a Stop cassette interposed between a *tk* gene and the strong constitutive cytomegalovirus immediate early gene promoter (CMV IE). Plasmid p*tk* was similar, but without the Stop cassette, i.e. a plasmid containing a *tk* gene under the direct control of a cytomegalovirus gene CMV IE promoter.

After transfection, cultures were irradiated 3-4hrs later, and after 5 days cell growth was determined, calculated in the presence and absence of 50  $\mu$ M GCV (ganciclovir) for all cell lines in independent experiments.

The results illustrated in Figure 9a and 9b show that the molecular switch is very efficient. Using the MCF-7/E4*cre* cell line as a control, (O in Figure 9a), similar increases in the extent of GCV-mediated growth inhibition were seen following irradiation of MCF-7 cells that had been transfected with the plasmid p*tk* in which the CMV promoter operated the expression of tk,



(graph ▼ in Figure 9a) and MCF-7-*cre* cells that had been transfected with pStk wherein the radiation-triggered molecular switch was required to achieve GCV-mediated sensitisation (graph ● in Figure 9a). In comparison, using MCF-7 cells as a control MCF-7 cells transfected with pE4*tk* (in which the synthetic radiation-responsive promoter E4 directly operated the expression of a *tk* gene) the increase in sensitisation following irradiation was considerably less (graph ■ in Figure 9b).

Since the switch system resulted in the same levels of sensitisation as an unswitched system, it is concluded that the amounts of Cre recombinase produced upon irradiation, even at the lowest dose used, were not a limiting factor in the effect. For clinical treatment of tumours, it would thus be an advantage to administer mixtures of small amounts of Cre-expressing plasmid together with larger amounts of tumour-sensitizing gene expressing plasmid or plasmids in order to obtain the maximum molecular switching effect and hence tumour sensitisation from a single dose of radiation.

It will be appreciated that the Stop cassette-containing plasmids may themselves be a mixture containing different tumour sensitizing genes and in practice a mixture most suitable for the tumour type being treated would be used. This would facilitate the manufacture of the plasmids since the cre-expressing plasmid would be common to any treatment protocol and would be combined with a stop cassette plasmid of various kinds such as would be most appropriate for the specific characteristics of the tumour in question.

#### Demonstration of the use of Platinum containing drugs as control gene expression inducing agents

As indicated earlier, it has also been found that genes having radiation responsive promoters or other expression regulatory elements can in many

cases respond also to, and be upregulated by, platinum containing drugs such as cisplatin (CDDP) for example. This is demonstrated in Figure 10 which shows the results obtained in experiments in which the expression was determined (by FACS analysis) of Green Fluorescent Protein (GFP) encoded in a plasmid  
5 vector pE4-*gfp* containing the *gfp* gene directly controlled by the synthetic radiation responsive promoter E4.

As shown in Figure 10, 1micromolar CDDP induced the expression of GFP to a similar extent as did ionising radiation (IR). Thus CDDP was able to induce gene expression regulated by the radiation-responsive elements in the  
10 promoter region upstream of the *gfp* gene. Hence this agent or, in principle, any similar agent or agents producing similar or effective DNA damage could be used to trigger the molecular switch and effect the expression of tumour sensitizing genes. It was noted that combinations of CDDP and IR were more effective than either of the treatments alone. Thus two or more agents may  
15 synergise and allow the switch effect to occur at even lower sub-toxic and sub-lethal doses of the individual inducing agents.

To use such platinum containing drugs, or indeed any chemical inducing agents of which there are many possibilities (given an appropriate choice of control gene promoter elements), to provide the required control gene  
20 expression inducing influence, it is anticipated that for clinical administration such agents would most probably be encapsulated into cationic liposomes with a view to tumour targeting. Thus, such targeted drugs may be used instead of radiation in the present invention. Importantly, and by analogy with the radiation-mediated gene therapy, the amounts of these chemical inducing  
25 agents that would be required to trigger the response would generally be less than those required to kill the tumour cells if the agents were to be used without the molecular switch.

### Demonstration of Control gene expression induced by Hypoxia

In these experiments the HIF-1 binding domain of the enolase-1 gene was produced as a double-stranded oligonucleotide by annealing of the synthetic oligonucleotides HRE1 and HRE2 (see Table 3). The annealed  
5 oligonucleotide was ligated into *Bgl* II-*Sgf* I digested pCIneo (Promega) to produce a plasmid designated pHCIneo. pHCIneo was digested with *Eco*RI and *Not*I and the *Eco*RI-*Not*I fragment of pEGF-1 (Clontec) that contains the GFP gene was ligated into the resulting gap to generate a vector pHRE-EGFP.

NB1G human neuroblastoma cells were plated in culture medium at  
10  $7.5 \times 10^4$  cells per well in a 24-well plate and incubated at 37°C in an atmosphere containing 20% oxygen. Three days later they were transfected with pEGFP (which contains no promoter), pCMVGFP (which contains the CMV promoter) or pHREGFP (which contains the hypoxia-responsive promoter) using as a transfecting agent Lipofectamine Plus as described by the  
15 manufacturer. After 24 hours, the medium was replaced with fresh medium or medium containing 25 micromolar  $\text{CoCl}_2$ . Following incubation overnight in an atmosphere containing 5%  $\text{O}_2$ , FACS analysis was performed as described elsewhere. These conditions were intended to produce hypoxia in the transfected neuroblastoma cells.

20 The results are illustrated in Figure 11 which shows that pHREGFP-transfected cells were more fluorescent than the control pEGFP or pCMVGFP cells under low (5%) oxygen conditions, whilst  $\text{CoCl}_2$  had no or only a slight inhibitory effect. However, in the latter two cases, with the pHREGFP-transfected cells there was a considerable increase in the number of fluorescent  
25 cells in the population. Thus it was shown that GFP expression from the pHREGFP vector can be induced by conditions of hypoxia.

### METHODS and MATERIALS - Summary

For completeness there now follows an outline or summary of some of the methods, techniques and materials which have generally been used in development and testing of this invention unless stated otherwise.

### Cells

5 All bacterial cloning was carried out in *E.coli* strain XL-1 blue MRF' (Stratagene).

The human cell line used for transfection was MCF-7 (breast carcinoma) obtained from the American Tissue Culture Collection (HTB No. 22). The DNA used for PCR amplification of the *egr1*-1 promoter elements was  
10 extracted from the human cervical carcinoma line, HeLa.

### Plasmids

The pCI and pCI-neo plasmids were obtained from Promega.

The pEGFP-1 plasmid was obtained from Clontech.

The pBS185 (containing the *cre* recombinase gene) and pBS302 (Stop  
15 cassette) plasmids were obtained from Gibco BRL™ (Life Technologies, Inc.).

The plasmid clone pBR-*tk* was produced by cloning the nucleotide sequence of the *Herpes simplex* virus type 1 (HSV) thymidine kinase (*tk*) gene, strain CL101. (described by Wagner *et al.*, (1981) as previously mentioned) into Gibco plasmid pBR322, using standard methods.

### 20 Enzymes

All restriction endonucleases were obtained from MBI Fermentas, with the following exceptions. *SgfI* and *I-Ppo-I* were from Promega. *AccI*, *AscI* and *SpeI* were from New England Biolabs.

T4 DNA ligase, Taq DNA polymerase and Klenow were from MBI  
25 Fermentas. Expand High Fidelity polymerase was from Boehringer Mannheim. Taq Plus Precision was from Stratagene.

All enzymes were used in the buffers supplied and in accordance with the manufacturers instructions.

#### DNA Purification

High molecular weight HeLa DNA was extracted from cultured cells using the MBI Fermentas Genomic G2 kit. Large-scale plasmid DNA preparations were carried out using the Qiagen Maxi-purification kit.

DNA extraction and purification from agarose gels (Seakem GTG agarose, FMC Bioproducts) was carried out using the Qiagen™ Gel Extraction kit.

After restriction endonuclease digestion or modification, DNA was routinely purified using the Nucleon PCR/Oligo Clean-up kit.

#### Nucleotides

Deoxynucleotides for PCR were obtained from Pharmacia and used at 0.2mM final concentration.

Synthetic oligonucleotides for providing the radiation responsive elements and PCR primers less than 60 base pairs in length were obtained from Gibco BRL™. Those of a greater length were obtained from Cruachem.

#### Transformation of *E.coli*

The procedure used to prepare competent cells for molecular transformation and freezing was that outlined in the previously mentioned reference of Sambrook *et al.* (1989), with a 10mL 0.1M magnesium chloride cell pellet resuspension step prior to the first calcium chloride stage to increase transformation efficiency. The transformation procedure itself was also from this source.

#### Growth and transfection of human cells

Pre-confluent human tumour cells (MCF-7 [ATCC HTB 22]) grown in RPMI tissue culture medium + 10% foetal calf serum (FCS) were washed with PBS, serum free media (SFM) and then exposed to a transfection mixture of 15µL lipofectamine (Gibco BRL™) mixed with 85µL double-distilled water (ddH<sub>2</sub>O) combined with 10µL (5µg) of plasmid DNA mixed with 90µL ddH<sub>2</sub>O. This addition was immediately followed by adding 800µL RPMI + 5% FCS and incubating at room temperature for 5 hours. Subsequently, 1mL of RPMI + 15% FCS was added for 21 hours, after which the medium was removed and replaced with fresh complete RPMI + 10% FCS.

#### 10 Irradiation

Cells were irradiated at 37°C with 5Gy and 10Gy (2 x 5Gy) of cobalt-60 (gamma-rays) at a dose rate of 1 Gy min<sup>-1</sup>.

#### FACS analysis

Green Fluorescent Protein (GFP) expression was assessed by fluorescent activated cell sorting (FACS) and scanning (Becton-Dickinson FACScan™: excitation 488 nm). Monolayers of cells were trypsinised, washed with PBS (phosphate buffered saline) and finally resuspended in PBS as a single cell suspension. FITC fluorescence was measured at 530 ± 15 nm, and cell debris resulting in forward and side scatter was gated out. The gated fluorescence 1 profile of the FACScan™ software was used to determine the number of fluorescent cells in the sample.

#### MTT assay

Cytotoxicity assays were performed using a modification of a method previously published (Morten *et al.*, 1992 "Upregulation of O<sup>6</sup>-alkyl-guanine-DNA-alkyltransferase expression and the presence of double minute chromosomes in alkylating agent selected Chinese hamster cells" *Carcinogenesis* 13(3): 483-487). Briefly, confluent cultures of cells were

trypsinised, twice washed in PBS and resuspended to 2000 cell/mL in complete tissue culture medium (RPMI + 10% foetal calf serum) containing 50 mM ganciclovir (GCV). Aliquots (200  $\mu$ L) of this suspension were pipetted into each well of 96 well microtitre plates. The plates were incubated at 37°C for 4 days, after which the medium was aspirated and replaced with 100  $\mu$ L of a 3 mg/mL solution of MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] in PBS for 3 hours, followed by 200  $\mu$ L of DMSO. The plates were agitated to ensure complete dissolution, and were read on a multiplate reader (Flow Ltd.) at 530 nm and 690 nm.

## 10 DNA Manipulations

### (a) *PCR:*

The *tk* ORF was obtained by PCR using the specific primers shown in TABLE 2. The proof-reading DNA polymerases, Expand High-fidelity and Taq Plus PCR were used to avoid introduction of mutations. After 2 minutes of initial DNA strand melting at 94°C, polymerase was added. Amplification was carried out for 15 cycles under the following conditions; 94°C 1 min, 55°C 1 min, 72°C 1min. The latter, elongation, step was extended by 20 seconds each cycle for the last 5 cycles. A single 5 min elongation was also added as a final step. Products amplified from plasmid DNA were agarose gel purified to remove the original template.

### (b) *DNA Restriction endonuclease digestion:*

As already described, a series of steps was needed to produce the pComplete1 construct. Briefly, to recap. and summarise:

- (i) The Stop cassette was excised from pBS302 with *Eco*RI and *Spe*I and was cloned into the *Eco*RI and *Xba*I sites of pCI-*neo* to produce pStop (see Figure 4).

- 5 (ii) The *tk* ORF was amplified from the HSV plasmid clone pBR-*tk*, using the *tk* PCR primer pair shown in TABLE 2. The purified PCR fragment was then treated with *Cla*I and *Not*I and cloned into *Acc*I/*Not*I digested pCI-*neo* to generate pStop-*tk* (also see Figure 4).
- (iii) The radiation-responsive *egr-1* elements (RR-elements(a)) were cloned into the pCI-*neo* plasmid as described, using the *Bgl*II and *Cgf*I sites to generate pEGRL(a)-*neo*.
- 10 (iv) The Cre recombinase ORF was excised from pBS185 with *Xho*I and *Mlu*I and cloned into those sites in pEGRL(a)-*neo* to generate pEGRL(a)-*cre*.
- (v) The *Not*I site was removed from the pEGRL(a)-*cre* plasmid by *Not*I digestion. This was followed by "filling-in" of single-stranded overhangs using Klenow, then re-ligation.
- 15 (vi) The *Bgl*II/*Bam*HI fragment of the pCI plasmid, containing the CMV I.E. enhancer/promoter, multiple cloning site and SV40 polyadenylation site, was isolated and then cloned into the *Not*I-deletion construct via partial *Bam*HI digestion, allowing insertion downstream of the neomycin resistance gene to generate
- 20 pEGRL-*cre*MCS2.
- (vii) Finally, the *Eco*RI/*Not*I Stop-*tk* cassette of pStop-*tk* was cloned into the inserted multiple cloning site in pEGRL-*cre*MCS2 to produce pComplete1.
- (c) *Ligation:*
- 25 For ligations involving only single-stranded terminal overhangs, 20-50ng of plasmid vector DNA was added to 60-200ng of potential insert DNA



and incubated at 21-25°C for approximately 20 hours before transformation. Blunt-end ligations were performed at 4°C.

The radiation responsive element containing oligonucleotides were annealed by placing 0.2-0.5nmols of each complementary molecule in a 5µL total volume and heating to 55°C for 5min before leaving to cool for about 24hrs. This mixture was then added to 50-100ng of plasmid vector for ligating as normal.

### CLINICAL APPLICABILITY

In clinical use the vector material of the present invention will be administered to a cancer patient in a suitable dose and in a suitable pharmaceutical composition using, as already explained, either a virus-based or virus-free method of DNA delivery.

After administration of the composition containing the vector to the patient an appropriate time is allowed for uptake and incorporation into cells. The patient's tumour may then be subjected to a suitable dose of diathermy, ionizing radiation or other appropriate exogenous inducing agent (unless relying on an endogenous inducing influence). In the case of ionizing radiation, this is preferably applied using conformal radiotherapy apparatus, or a tumour-targeting radiolabelled agent such as a tumour specific radio labelled antibody or cytokine may be used. In the case that the tumour has metastasised, when appropriate whole body diathermy or radiation or a tumour-targeting radiolabelled agent may be used. The inducing agent activates the inducing agent responsive promoter of the control gene with the result that recombinase protein is expressed. Although this expression may be transient, the action of the recombinase results in the recombination of the recombinase target sites and hence excision of the Stop cassette, resulting in the permanent expression of the tumour-sensitizing gene or genes as hereinbefore described in connection with the specific embodiments referred to.

When a prodrug activating enzyme is encoded by the sensitizing gene(s), usually after administration of the vector composition in the course of clinical use and application of heat or ionizing radiation or other inducing agent as the case may be, an appropriate therapeutically effective dose of the prodrug  
5 is administered in a conventional formulation by any suitable route. This then brings about the death of the cells that express this prodrug activating enzyme, and also cells in the vicinity by virtue of the bystander effect.

In an alternative, if the tumour killing or sensitizing gene encodes proteins such as for example interleukin-2 (IL-2) or granulocyte-macrophage  
10 colony-stimulating factor, an immune response will be stimulated in the host that should eradicate the tumour cells. On the other hand, if the tumour killing or sensitizing gene encodes a cytotoxic protein, e.g. ricin, expression can result directly in the killing of the host tumour cells.

In the case that the tumour sensitizing gene encodes a ribozyme or an  
15 antisense RNA molecule, these would be designed to bind to and cleave, or elicit the cleavage of, specific messenger RNA molecules that encode proteins the non-production of which would lead to the death of the cells or render the cells more sensitive to killing by exogenous agents or ionising radiation. However, with this approach it is likely that only those cells to which the  
20 therapeutic DNA is delivered would be killed or sensitised, whereas with prodrug activation bystander effects can occur.

The advantages of the invention include the fact that in some embodiments using diathermic heat or ionizing radiation as exogenous inducing agents recombination and subsequent activation of the vector material only  
25 occurs in the region of diathermy or in the irradiated area. The uptake of the vectors into normal cells at distant sites will not therefore result in the ability of such cells to activate prodrugs or otherwise elicit tumour sensitisation. Furthermore, very low sub-therapeutic doses of ionizing radiation can be

effective in inducing expression of, for example, the *egr-1* enhancer/promoter. Hence, in the case that the tumour has metastasised, tumour-seeking radiolabelled agents that are inefficient in killing tumour cells *per se* can be used to instigate the recombination events and enable the activation of  
5 prodrugs, or the other cancer cell-killing processes described, in potentially all metastatic sites as well as in the primary tumour.

As will be seen, the invention presents a number of different aspects and it should be understood that it embraces within its scope all novel and  
10 inventive features and aspects herein disclosed, either explicitly or implicitly and either singly or in combination with one another. Also, many detail modifications are possible and, in particular, the scope of the invention is not to be construed as being limited by the illustrative example(s) or by the terms and expressions used herein merely in a descriptive or explanatory sense.

TABLE 1

Synthetic single-stranded oligonucleotides used to produce double-stranded molecules containing radiation responsive elements (RRelements) E provided by repeats of the decamer: 5' CCTTATTTGG (SEQ ID NO:1).

- 5 Plasmid pComplete1 contains a total of 6 elements (E6) arranged in series in a tandem array:

EGRE 1

*Bgl*II(part of)

5' GATCTCCTTA TTTGGCCTTA TTTGGCCTTA TTTGGCCTTA  
10 TTTGGCCTTA TTTGGCCTTA TTGGGCGAT

*Sgf*I(part of)

(SEQ. ID NO: 2)

EGRE 2 (complementary to EGRE1)

5' CGCCCAAATA AGGCCAAATA AGGCCAAATA  
15 AGGCCAAATA AGGCCAAATA AGGCCAAATA AGGA

(SEQ. ID NO:3)

Examples of similar oligonucleotides containing four repeats (E4) of SEQ. ID NO: 1

20 EGRE 3

*Bgl*II (part of)

5' GATCTTTATT TGGCCTTATT TGGCCTTATT TGGCCTTATT  
TGGGCGAT

*Sgf*I(part of)

25

(SEQ. ID NO: 4)

EGRE 4 (complementary to EGRE 5)

5' CGCCCAAATA AGGCCAAATA AGGCCAAATA  
AGGCCAAATA AGGA

30

(SEQ. ID NO: 5)

TABLE 2

A. Primers used for PCR amplification of promoter/enhancer sequences of human primary response gene *egr-1*/TIS8.

For enhancer/promoter:

5 EGRE 5

*Bgl*II

5' TCCAGATCTC CCGGTTGCT CTCACGGTCC CTGAGG

(SEQ. ID NO: 6)

EGRP 6

10

*Asc*I

5' CGGCGCGCCG CTGGATCTCT CGCGACTCCC CG

(SEQ. ID NO: 7)

For enhancer alone:

EGRE 7

15

*Sgf*I

5' ACTGCGATCG CGGGCCCGGC CCGGCCCGCA TCCCAGGCCC  
CC

(SEQ. ID NO: 8)

B. Primers used for PCR amplification of Thymidine kinase gene

20 Clark:

*Cl*aI

5' CCATCGATAT GGCTTCGTAC CCCGGC

(SEQ. ID NO: 9)

tkNot:

25

*Not*I

5' AAGGAAAAAA GCGGCCGCCT CCTTCCGTGT TTCAGTTAGC

(SEQ. ID NO: 10)

TABLE 3

Synthetic single-stranded oligonucleotides used to produce double-stranded molecules containing the hypoxia responsive region of the Enolase-1  
5 gene promoter.

HRE1

*Bgl* II (part of)

5' GATCTAGGGC CGGACGTGGG GCCCCGTAGG CACGCTGAGT  
10 GCGTGCGGGA CTCGGAGTAC GTGACGGAGC CCCGCGATGC  
GAT

*Sgf* I (part of)

(SEQ. ID NO:11)

15

HRE2 (complementary to HRE1)

5' CGCATCGCGG GGCTCCGTCA CGTACTCCGA GTCCCGCACG  
CACTCAGCGT GCCTACGGGG CCCACGTCC GGCCCTA

(SEQ. ID NO:12)

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CLAIMS

1. Vector material characterised in that said material contains:
  - (a) a tumour cell sensitizing gene or genes of which expression in a tumour cell yields a sensitizing gene expression product having a potential to cause tumour cells to be killed and destroyed, or to be eliminated, or otherwise to be inactivated, or to be rendered sensitive and/or vulnerable to destruction;
  - (b) a sensitizing gene expression regulatory system, including promoter means, for said sensitizing gene or genes;
  - (c) at least one control gene; and
  - (d) a control gene expression regulatory system responsive in use in a transfected cell to the effect of a predetermined exogenous or endogenous expression inducing influence so as to induce expression of said control gene to yield an expression product having a capacity to establish an operative linkage between said sensitizing gene expression regulatory system and said sensitizing gene or genes effective to trigger and switch on or permit continuous or permanent expression of the latter to bring about continuous production of said sensitizing gene expression product.
2. Vector material as claimed in Claim 1 wherein the tumour sensitizing gene expression product is an enzyme or other bioactive agent that can convert a predetermined inactive prodrug into an active cytotoxic drug.
3. Vector material as claimed in Claim 2 wherein the tumour-sensitizing expression product is a prodrug activating enzyme selected from the following:
  - HSV thymidine kinase, nitroreductase, cytosine deaminase, cytochrome p450 2E1 or cytochrome p450 2DVI.

4. Vector material as claimed in Claim 1 wherein the tumour-sensitizing expression product is an immune-response modifying agent selected from the expression products of the following:

GM-CSF, IFN-alpha, IFN-beta, IFN-gamma, IL-1beta, IL-2, IL-4, IL-6,  
5 IL-8, IL-10, IL-12, IL-15, TNFalpha.

5. Vector material as claimed in any one of the preceding claims wherein the control gene encodes a recombinase enzyme that acts on recombinase target sites to modify the vector material to establish said operative linkage between the sensitizing gene expression regulatory system and the sensitizing gene or  
10 genes.

6. Vector material as claimed in Claim 5 wherein the control gene and the recombinase target sites are part of a Cre-loxP or a FLP-fRT site specific recombination system.

7. Vector material as claimed in Claim 5 or 6 wherein said recombinase  
15 target sites are separated by a region containing a "stop" sequence of nucleotides that blocks or otherwise prevents expression of the sensitizing gene or genes until removed by the action of said recombinase enzyme.

8. Vector material as claimed in Claim 5 or 6 wherein the protein coding regions of the sensitizing gene or genes are operationally separated from the  
20 said promoters and wherein said recombinase target sites are arranged such that recombination brings about the juxtapositioning of the sensitizing gene promoters and protein coding regions of the sensitizing gene or genes resulting in their expression.

9. Vector material as claimed in any one of Claims 5 to 8 wherein the  
25 control gene is a fusion gene that when expressed produces a fusion protein consisting of a recombinase and an intercellular trafficking protein (such as for example the virion protein VP22).

10. Vector material as claimed in any one of Claims 5 to 9 wherein the region between said recombinase target sites contains a duplicate copy of the recombinase control gene together with an associated promoter.
11. Vector material as claimed in any one of the preceding claims wherein  
5 the sensitizing gene expression regulatory system incorporates at least one expression inducible element responsive to the effect of a predetermined exogenous or endogenous expression inducing influence.
12. Vector material as claimed in any one of the preceding claims wherein the sensitizing gene is a fusion gene that when expressed produces a fusion  
10 protein consisting of a sensitizing protein and an intercellular trafficking protein.
13. Vector material as claimed in any one of Claims 1 to 8 wherein the or each tumour sensitizing gene is selected from the *E.coli* nitroreductase gene, cytosine deaminase (CD) gene, *Herpes simplex* virus thymidine kinase (HSV-  
15 *tk*), mammalian cytochrome p450 2E1 or 2DVI gene, and their functional equivalents.
14. Vector material as claimed in any one of the preceding claims wherein the tumour cell sensitizing gene or genes and the control gene are in separate vectors.
- 20 15. Vector material as claimed in any one of Claims 1 to 13 wherein the tumour cell sensitizing gene or genes and the control gene are in the same vector.
16. Vector material as claimed in any one of the preceding claims for use in antitumour therapy characterised in that the use comprises the introduction of  
25 the material into tumour cells.
17. Vector material as claimed in Claim 16 wherein said expression inducing influence is endogenous and tumour related, being produced by

tumour cells associated specifically with tumours to which said antitumour therapy is directed.

18. Vector material as claimed in Claim 17 further characterised in that at least one element of the control gene expression regulatory system is selected  
5 so that the control gene is automatically upregulated to an effective operational level when the vector material is introduced into cells of said tumours.

19. Vector material as claimed in Claim 18 wherein the selection of said at least one element of the control gene expression regulatory system is carried out using gene array technology.

10 20. Vector material as claimed in any one of Claims 17 to 19 wherein said control gene expression regulatory system responds in use in a transfected cell to said expression inducing influence where the latter is provided by a change in environmental thermal conditions in cells containing the vector material.

21. Vector material as claimed in any one of Claims 17 to 19 wherein said  
15 control gene expression regulatory system responds in use in a transfected cell to said expression inducing influence where the latter is provided by a change in local oxygen concentration.

22. Vector material as claimed in any one of Claims 16 to 19 wherein said control gene expression regulatory system includes an expression control  
20 element responsive in use in a transfected cell to a hypoxia condition in the cellular environment.

23. Vector material as claimed in Claim 16 wherein said control gene expression regulatory system responds in use in a transfected cell to said expression inducing influence where the latter is provided by an exogenous  
25 expression inducing agent applied to cells into which the vector material is introduced.

24. Vector material as claimed in Claim 23 wherein the dose level of said exogenous expression inducing agent needed to trigger and switch on expression of said sensitizing gene or genes in the cells of tissue into which the vector material has been introduced is substantially sub-lethal.
- 5 25. Vector material as claimed in Claim 23 or 24 wherein said control gene expression regulatory system responds in use in a transfected cell to said expression inducing agent which is provided by at least one of the following:
- 10 electromagnetic radiation, application of heat or cooling, application of a magnetic or electric field, an exogenous chemical inducing agent, radiation in the form of sub-atomic particles.
26. Vector material as claimed in Claim 25 wherein said expression inducing agent is electromagnetic radiation in the form of ultra-violet or visible light.
- 15 27. Vector material as claimed in Claim 25 wherein said expression inducing influence is electromagnetic radiation in the form of X-rays or gamma-rays.
28. Vector material as claimed in Claim 25 wherein said expression inducing agent is electromagnetic radiation in the form of X-rays or gamma-rays at a substantially sub-lethal dosage.
- 20 29. Vector material as claimed in Claim 23 or 24 wherein said expression inducing agent is an exogenous chemical expression inducing agent that induces cellular damage.
30. Vector material as claimed in Claim 23 or 24 wherein said expression inducing agent is an exogenous chemical expression inducing agent in the form of an antitumour drug.
- 25

31. Vector material as claimed in Claim 30 wherein the antitumour drug is a platinum containing drug.

32. Vector material as claimed in Claim 30 wherein the antitumour drug is selected from the following:

5 Temozolomide, Dacarbazine, Streptozotocin, Procarbazine, Carmustine, Semustine, Lomustine, Fotemustine, Busulphan, Treosulphan, Mechlorethamine, Cyclophosphamide, Iphosphamide, Chlorambucil, Melphalan, ethyleneimines triethylene melamine, hexamethylmelamine, TEPA and thio-TEPA, dibromomannitol and dibromodulcitol,  
10 hydroxyurea, Methotrexate, azaserine Azathioprin, 5-azacytidine, 5-fluorouracil, cytosine arabinoside, 6-mercaptopurine, Allopurinol 6-thioguanine, deoxycoformycin, Tiazofurin, Acivicin, Pyrazofurin and p-aminolaevulinic acid, plant alkaloids such as Vinblastine, Vincristine and Vindesine, Etoposide and Teniposide, antitumour antibiotics such as  
15 Doxorubicin, Daunorubicin, Actinomycin, Bleomycins, Mytomycin, Mythramycin, Mitozantrone hormones such as oestrogen and progesterone.

33. Vector material as claimed in Claim 23 or 24 wherein said expression inducing agent is an exogenous chemical inducing agent in the form of a  
20 hormone that interacts with a receptor molecule which interacts with a complementary hormone responsive element in the control gene expression regulatory system.

34. Vector material as claimed in Claim 23 or 24 wherein the control gene expression regulatory system comprises a gene upregulation system that can be  
25 activated by a chemical agent.

35. Vector material as claimed in Claim 22 or 23 containing number of different control gene expression regulatory elements responsive to different



expression inducing influences so as to be activated under a range of different conditions.

36. Vector material as claimed in any of the preceding claims wherein at least one element of the sensitizing gene expression regulatory system is  
5 inducible in response to the effect of a predetermined exogenous or endogenous expression inducing influence.

37. Vector material as claimed in Claim 36 wherein the sensitizing gene expression regulatory system includes an expression control element responsive in use in a transfected cell to a hypoxic condition in the cellular environment.

10 38. Vector material as claimed in any of the preceding claims wherein at least one element of the sensitizing gene expression regulatory system is selected for efficiency in the particular tumour(s) to which said antitumour therapy is directed, the selection being carried out using gene array technology.

39. Vector material as claimed in any of the preceding claims which  
15 includes a plurality of tumour sensitizing genes providing a range of different expression products.

40. Vector material as claimed in Claim 39 wherein the different expression products are prodrug activating agents which are effective in relation to a range of different prodrugs, or which provide a range of different immune system  
20 stimulatory factors.

41. A pharmaceutical composition comprising vector material as claimed in any of the preceding claims in association with a pharmaceutically acceptable carrier or excipient.

42. A pharmaceutical composition as claimed in Claim 41 which further  
25 includes a transfection agent.

43. A kit comprising one or more unit doses of vector material as defined in any one of Claims 1 to 40 together with a transfection agent.

44. A kit comprising:
- (a) a vector which comprises a tumour cell sensitizing gene or genes and a sensitizing gene expression regulatory system as defined in any one of the Claims 1 to 40;
  - 5 (b) a vector which comprises a control gene and a control gene expression regulatory system as defined in any one of Claims 1 to 40; and
  - (c) instructions for the use of vectors (a) and (b) in antitumour therapy.
45. A kit comprising:
- 10 (a) vector material as defined in any one of Claims 1 to 40;
  - (b) a vector which comprises a tumour cell sensitizing gene or genes and a sensitizing gene expression regulatory system as defined in any one of Claims 1 to 40.
46. A kit as claimed in Claim 44 or 45 for use in antitumour therapy.
- 15 47. A kit as claimed in Claim 43, 44, 45 or 46 wherein each of the vectors and/or vector material is provided in the form of a pharmaceutical composition in association with a pharmaceutically acceptable carrier or excipient.
48. A kit as claimed in any one of Claims 43 to 47 wherein the sensitizing gene(s) produce(s) a prodrug activating agent and wherein one or more doses of
- 20 a prodrug matched to said prodrug activating agent are also included in said kit.
49. A method of treatment for cancer patients wherein there is delivered to tumour cells vector material as claimed in any of Claims 1 to 40, said cells then being subjected to the appropriate expression inducing influence.
50. A method of treating tumour cells in a biological host system
- 25 comprising:

- 5 (a) administering to the system an effective amount of a composition comprising vector material containing a tumour cell sensitizing gene or genes and having a control gene expression regulatory system responsive to a predetermined exogenous or endogenous expression inducing influence, said control gene expression regulatory system being operatively linked to a recombinase gene, together with recombinase target sites flanking a region of which removal permits continuous expression of said tumour cell sensitizing gene(s) as specified above, or primes said sensitizing gene(s) for continuous induced expression;
- 10 (b) causing said tumour cells transfected with said vector material to be subjected to a dose of an expression inducing agent effective to activate the recombinase gene expression control system of said vector material, thereby to bring about, via recombinase-mediated site specific recombination within the vector material, expression of
- 15 the or each tumour cell sensitizing gene component;
- and, in the case of prodrug activating sensitizing genes,
- 20 (c) administering to the host an effective amount of a composition comprising a prodrug convertible into an active form by the expression product of said tumour cell sensitizing gene or genes.

51. Use of vector material as defined in any one of Claims 1 to 40 in the manufacture of a medicament or of a kit as defined in Claim 43 or 45 for use in antitumour therapy.

52. Use of a vector which comprises a tumour cell sensitizing gene or genes and a sensitizing gene expression regulatory system as defined in any one of Claims 1 to 40 in the manufacture of a kit as defined in Claim 44 for use in antitumour therapy.

53. Use of a vector which comprises a control gene and a control gene expression regulatory system as defined in any one of Claims 1 to 40 in the manufacture of a kit as defined in Claim 44 or 45 for use in antitumour therapy.

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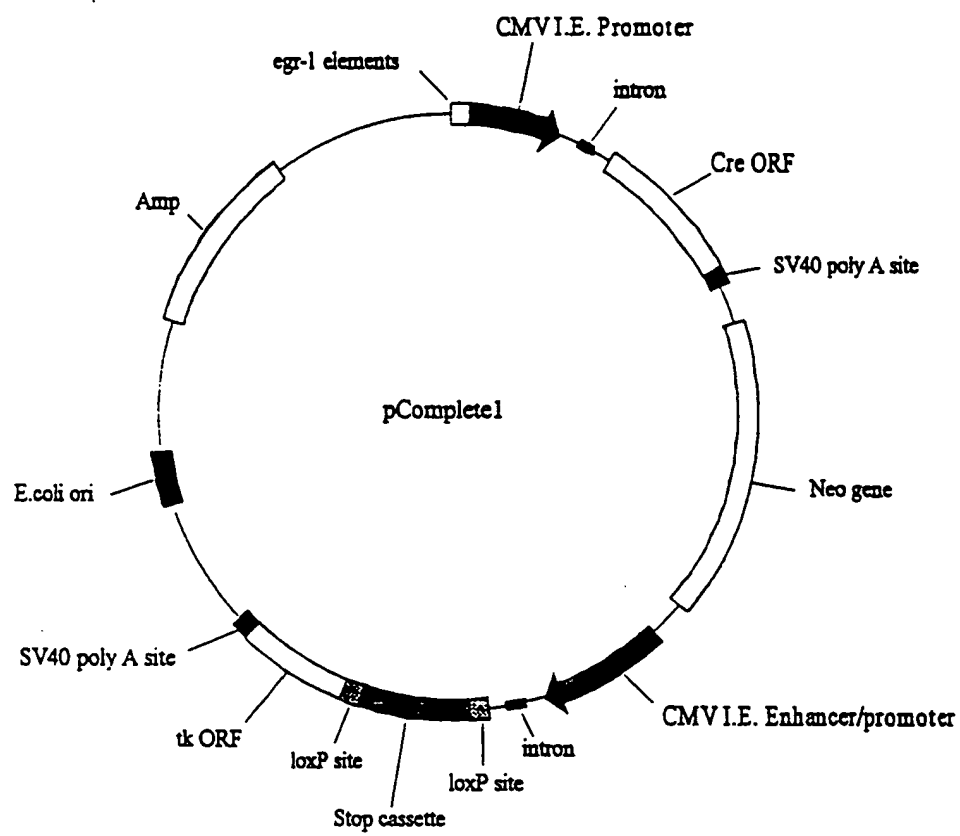


Figure 1.

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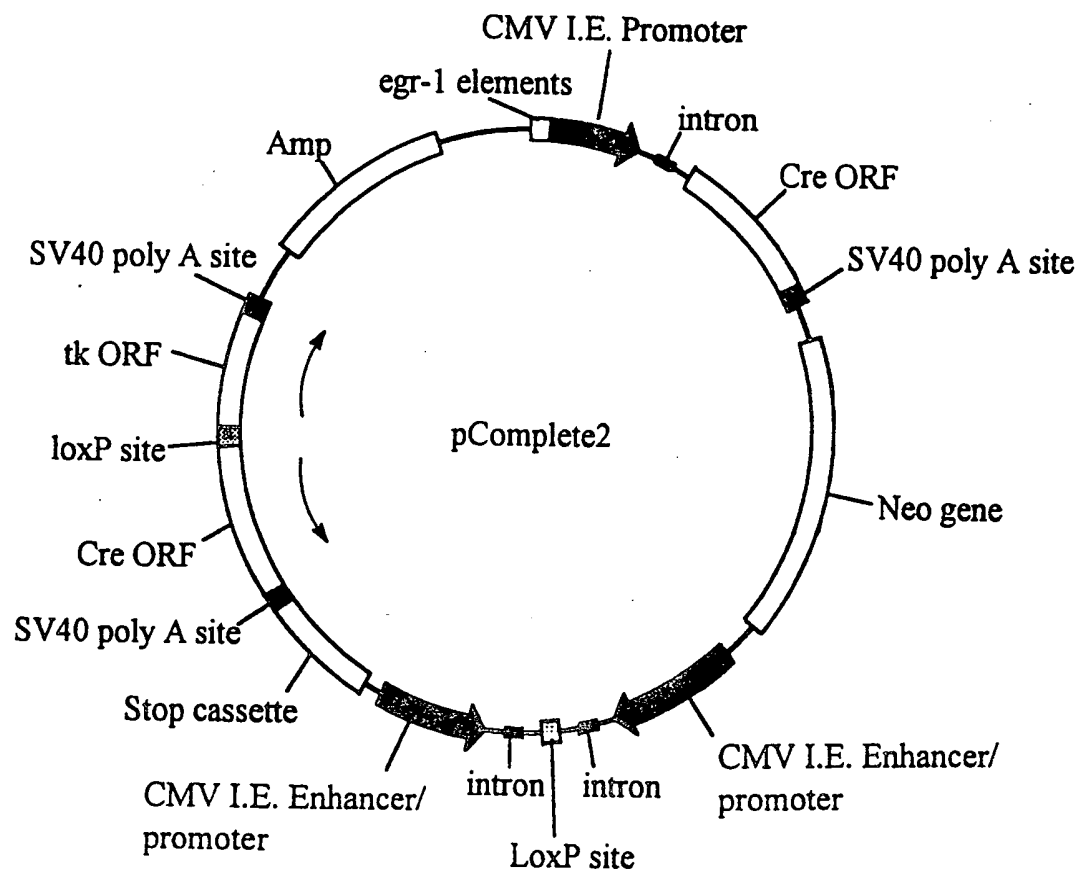


Figure 2.

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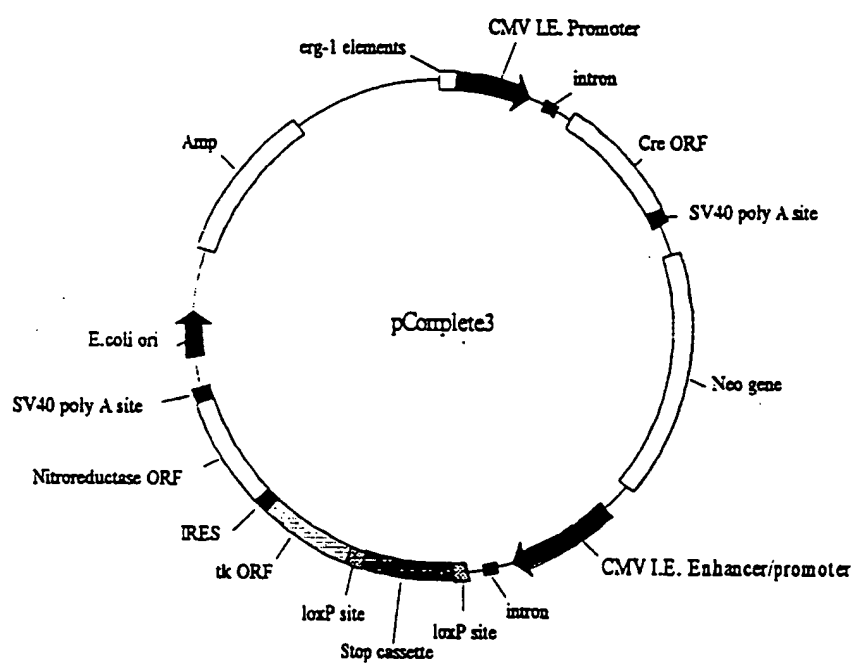


Figure 3.

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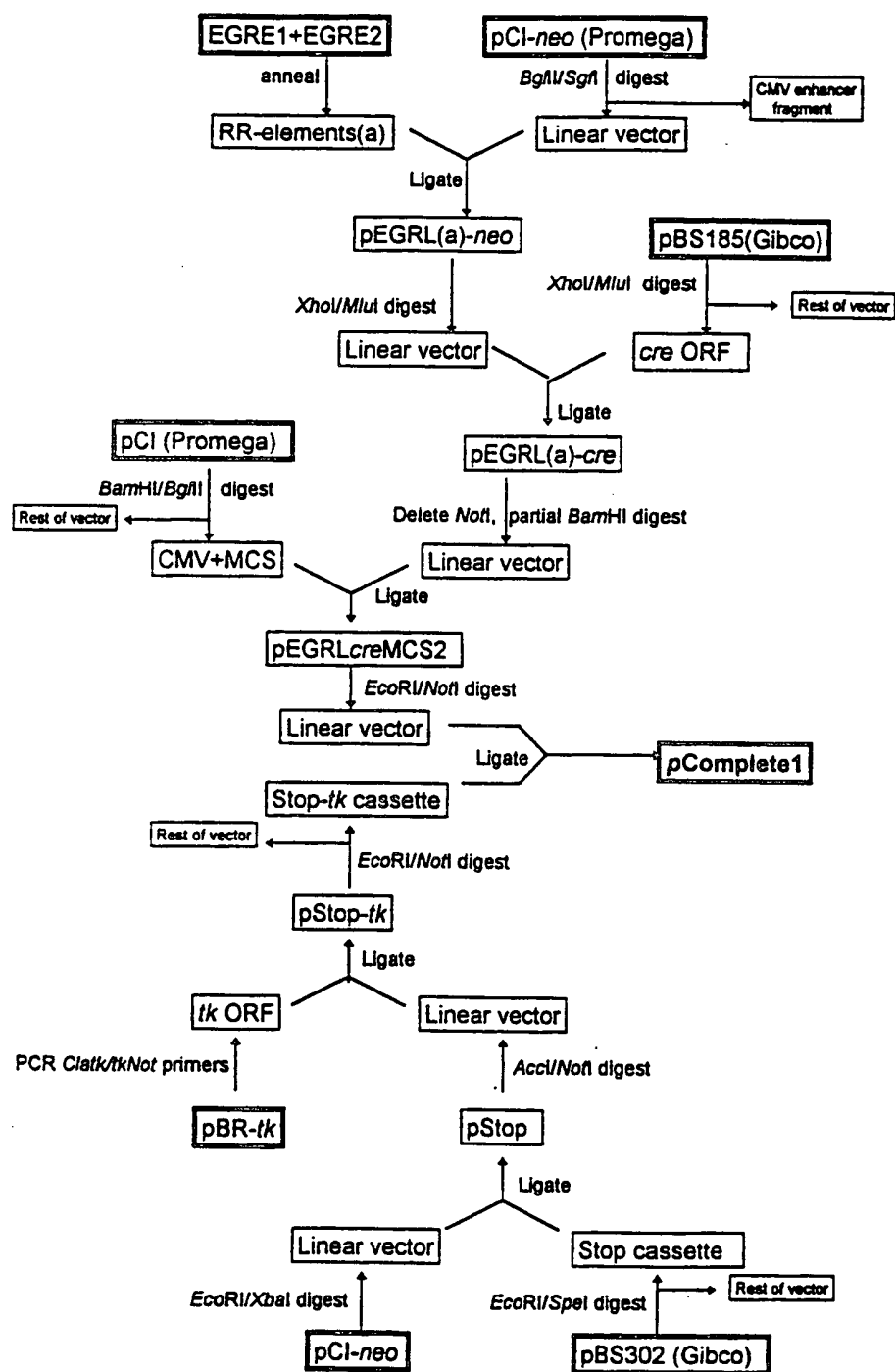


Figure 4.



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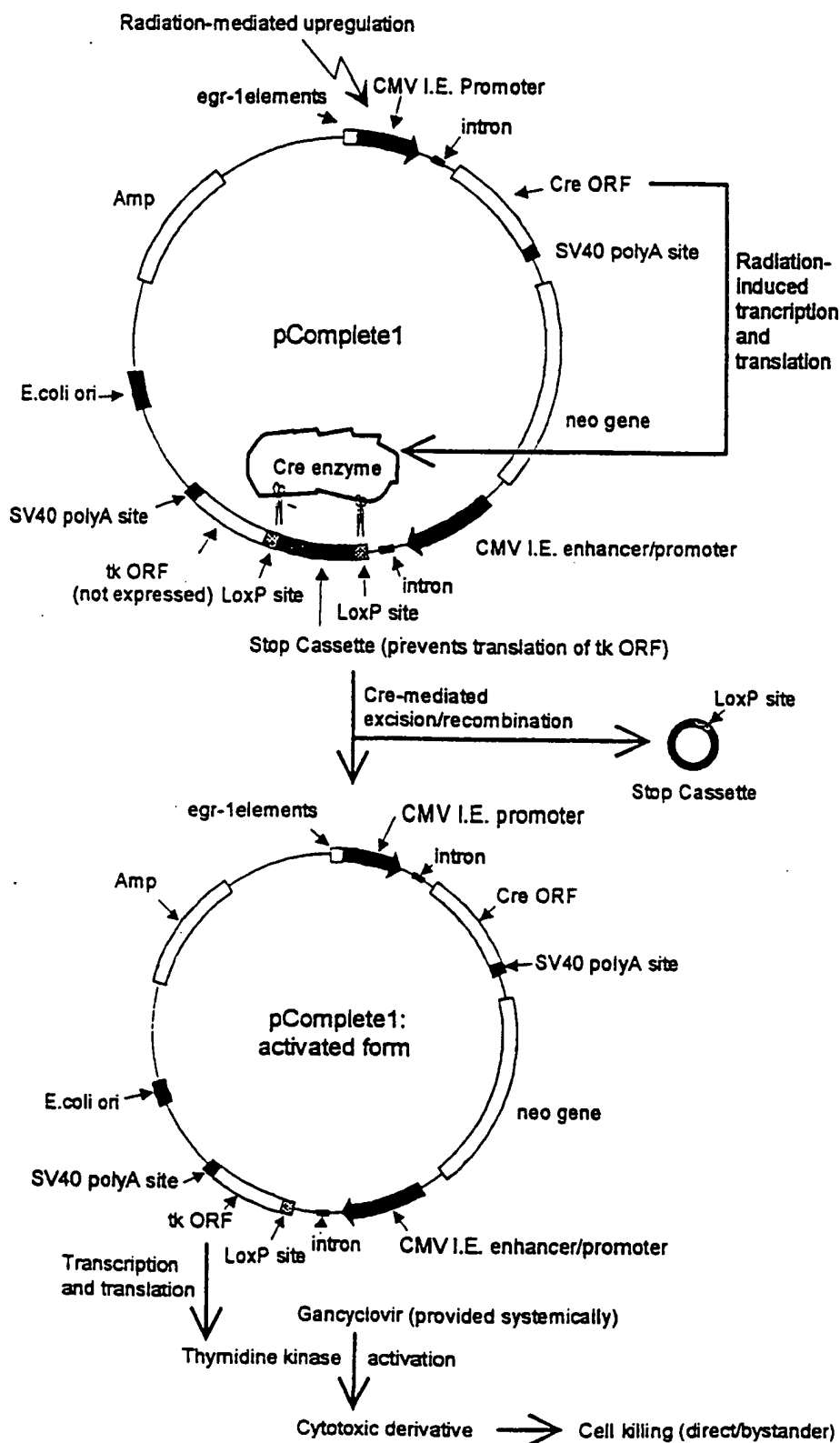


Figure 5.

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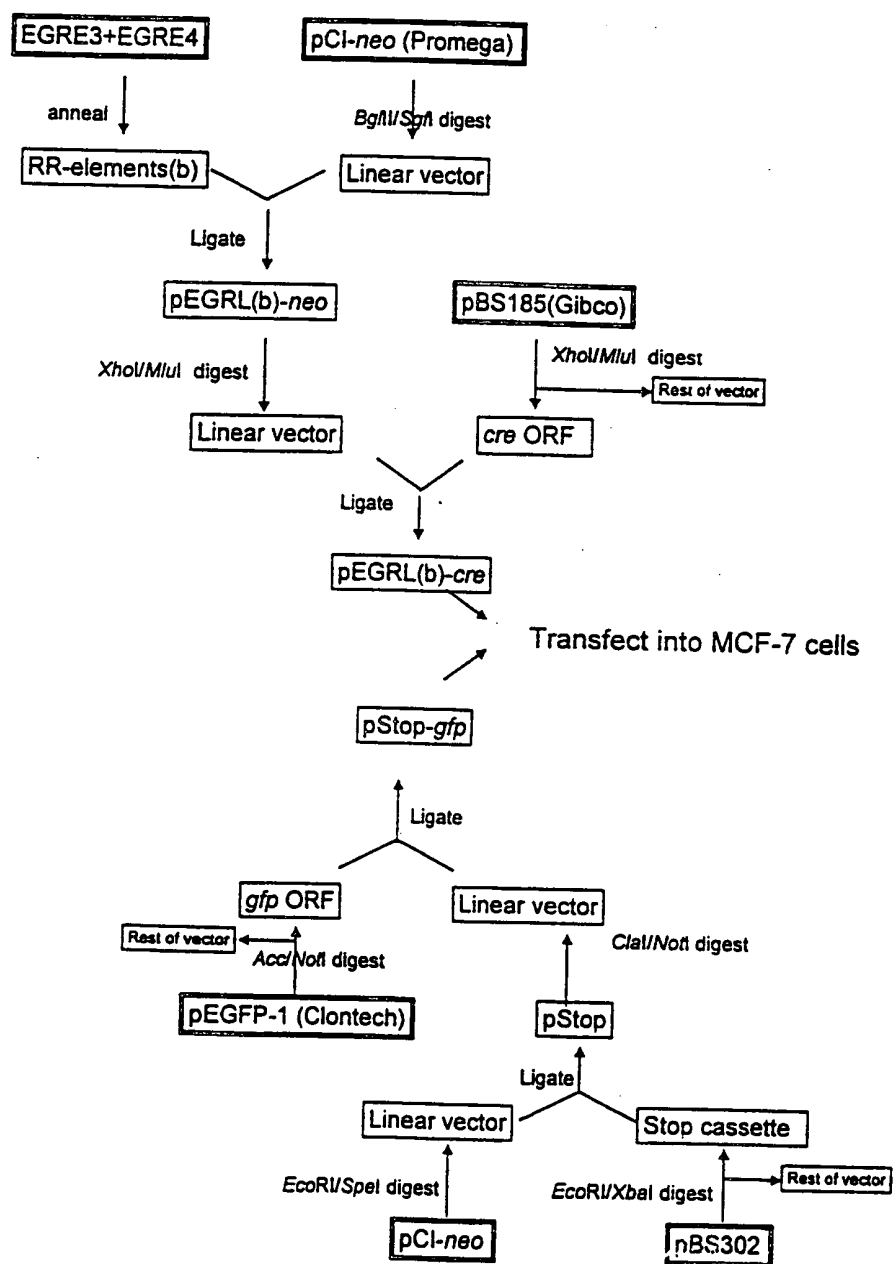


Figure 6.

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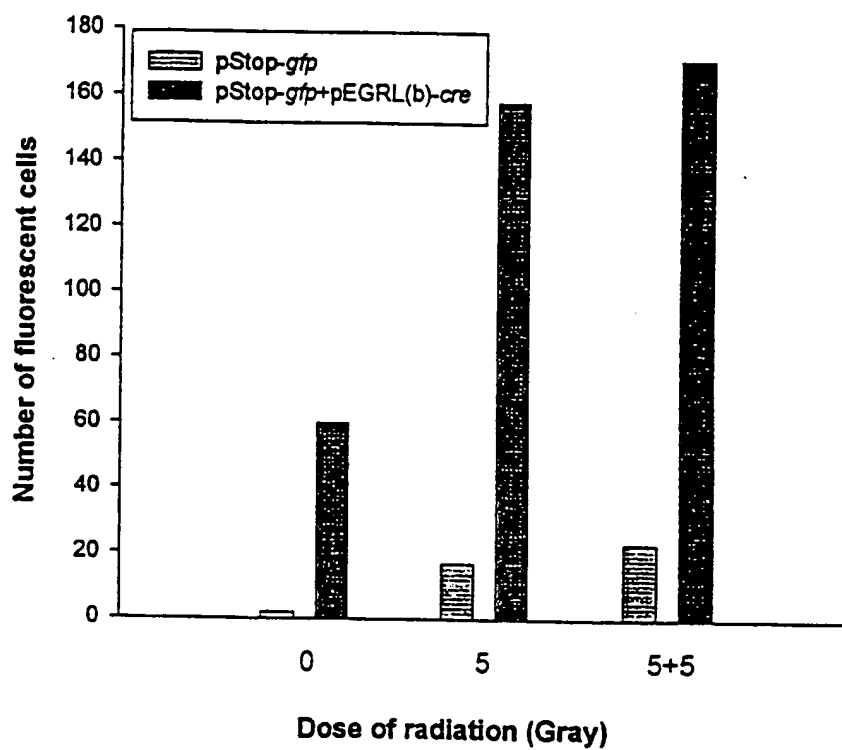


Figure 7.

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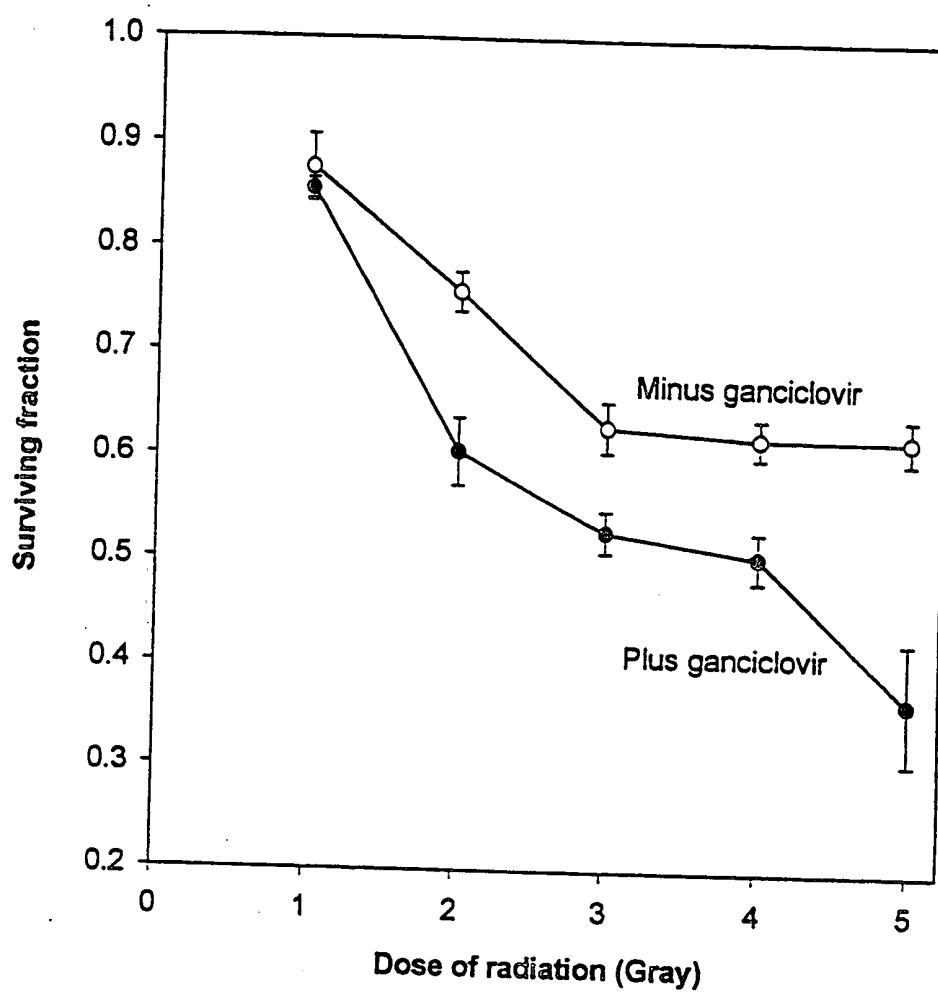


Figure 8.

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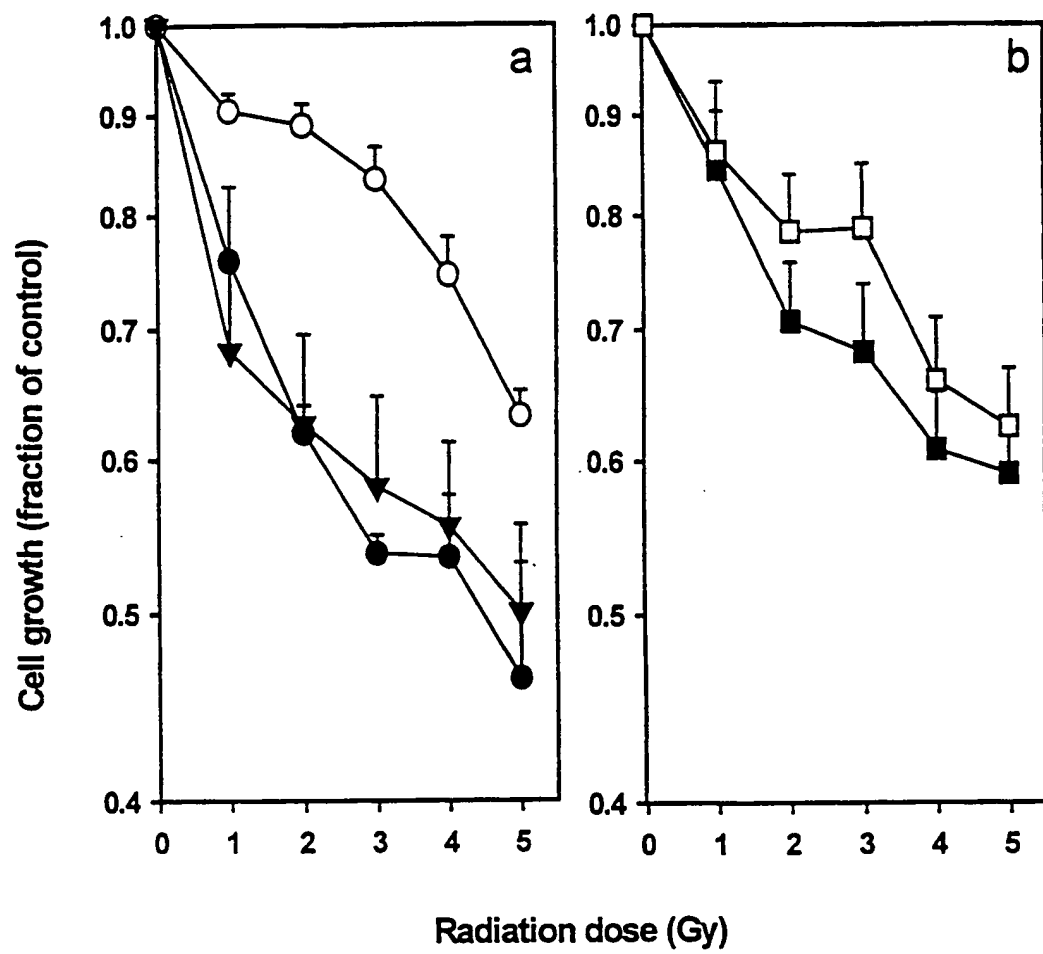


Figure 9a and 9b

10/11

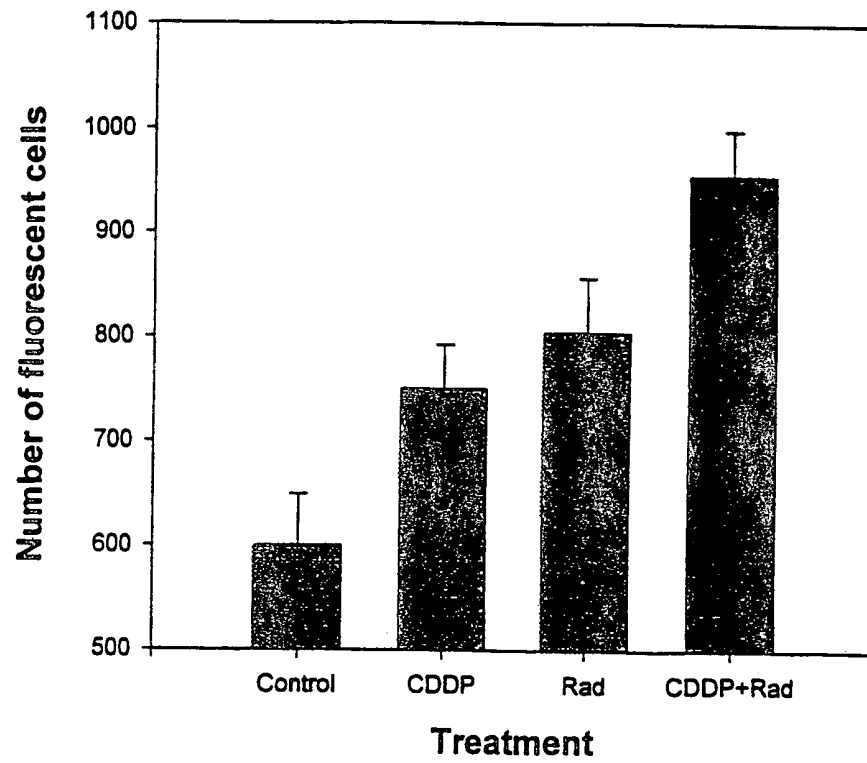


Figure 10.

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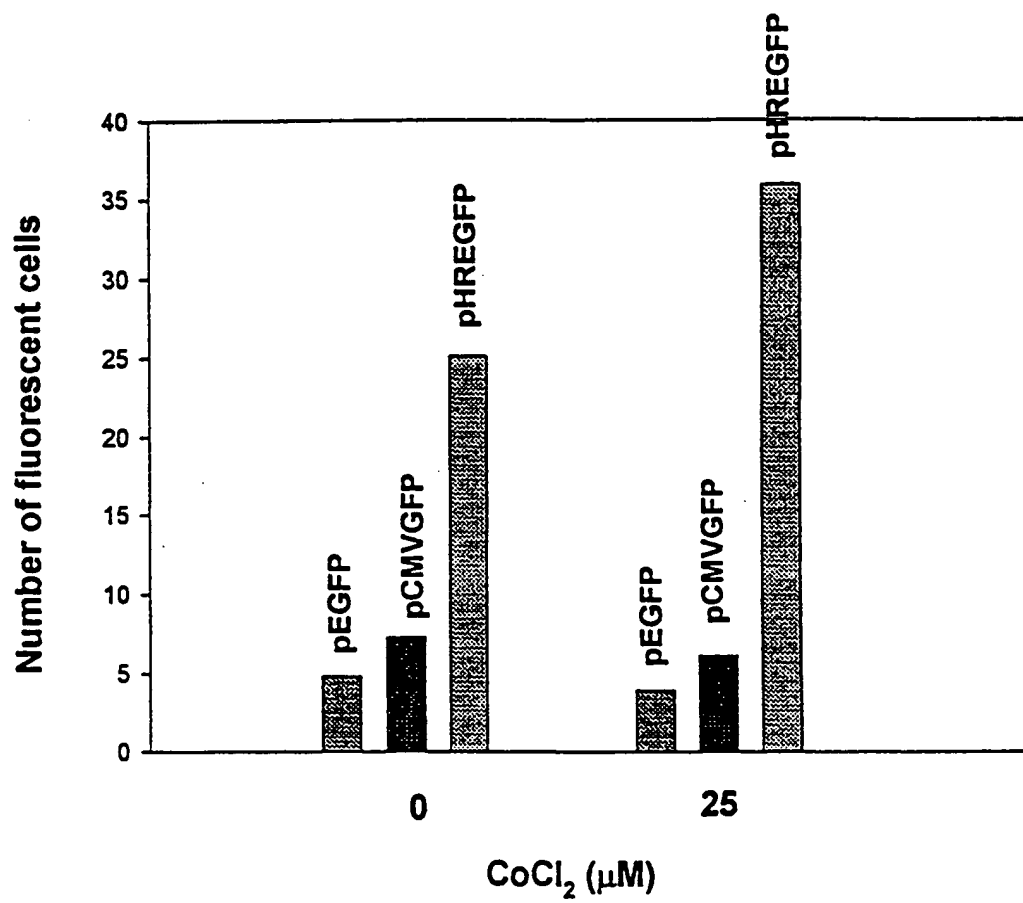


Figure 11.

SEQUENCE LISTING5    (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED  
      (B) STREET: CAMBRIDGE HOUSE, 6-10 CAMBRIDGE TERRACE,  
10                REGENT'S PARK,  
      (C) CITY: LONDON  
      (E) COUNTRY: UNITED KINGDOM  
      (F) POSTAL CODE (ZIP): NW1 4JL

- 15       (ii) TITLE OF INVENTION:  
          IONIZING RADIATION OR DIATHERMY-SWITCHED GENE  
          THERAPY VECTORS AND THEIR USE IN ANTITUMOUR THERAPY

- 20       (iii) NUMBER OF SEQUENCES: 12

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk  
      (B) COMPUTER: IBM PC compatible  
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
25       (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

30    (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs  
      (B) TYPE: nucleic acid  
35       (C) STRANDEDNESS: single  
      (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40    CCTTATTTGG

10

45    (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs  
      (B) TYPE: nucleic acid  
50       (C) STRANDEDNESS: single  
      (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

55    GATCTCCTTA TTTGGCCTTA TTTGGCCTTA TTTGGCCTTA TTTGGCCTTA TTTGGCCTTA 60  
      TTGGGCGAT 69

60



(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 64 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGCCCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA 60  
AGGA 64

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 48 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GATCTTTATT TGGCCTTATT TGGCCTTATT TGGCCTTATT TGGGCGAT 48

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGCCCAAATA AGGCCAAATA AGGCCAAATA AGGCCAAATA AGGA 44

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCCAGATCTC CCGGTTGCT CTCACGGTCC CTGAGG 36

(2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

10 CGGCGCGCCG CTGGATCTCT CGCGACTCCC CG

32

15

(2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 42 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

25 ACTGCGATCG CGGGCCCGGC CCGGCCCGCA TCCCAGGCCC CC

42

30

(2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

40 CCATCGATAT GGCTTCGTAC CCCGGC

26

45

(2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
55 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AAGGAAAAAA GCGGCCGCCT CCTCCGTGT TTCAGTTAGC

40

(2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 83 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GATCTAGGGC CGGACGTGGG GCCCCGTAGG CACGCTGAGT GCGTGCGGGA 50

15 CTCGGAGTAC GTGACGGAGC CCCGCGATGC GAT 83

20 (2) INFORMATION FOR SEQ ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 77 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CGCATCGCGG GGCTCCGTCA CGTACTCCGA GTCCCGCAGG CACTCAGCGT GCCTACGGGG 60

CCCCACGTCC GGCCCTA 77



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification:</b> <b>C12N 15/85, A61K 48/00,</b> <b>A61P 35/00, C12N 9/00,</b> <b>C12N 9/02, C12N 15/52,</b> <b>C12N 15/53, C12N 15/86</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 99/60142</b> <b>(43) International Publication Date:</b> 25 November 1999 (25.11.1999)
<b>(21) International Application Number:</b> PCT/GB99/01362 <b>(22) International Filing Date:</b> 17 May 1999 (17.05.1999) <b>(30) Priority Data:</b> 9810423.5 15 May 1998 (15.05.1998) GB <b>(60) Parent Application or Grant</b> CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [/]; (). MARGISON, Geoffrey, Paul [/]; (). MARPLES, Brian [/]; (). SCOTT, Simon [/]; (). HENDRY, Jolyon, Hindson [/]; (). MARGISON, Geoffrey, Paul [/]; (). MARPLES, Brian [/]; (). SCOTT, Simon [/]; (). HENDRY, Jolyon, Hindson [/]; (). WILSON GUNN SKERRETT; ().		<b>Published</b>
<b>(54) Title: GENE THERAPY VECTORS AND THEIR USE IN ANTITUMOUR THERAPY</b> <b>(54) Titre: VECTEURS DE THERAPIE GENIQUE ET UTILISATION DE CES DERNIERS DANS UNE THERAPIE ANTITUMORALE</b>		
<b>(57) Abstract</b> <p>Vector material useful for antitumour therapy contains: (a) a tumour cell sensitizing gene or genes of which expression in a tumour cell yields a sensitizing gene expression product having a potential to cause tumour cells to be killed and destroyed, or to be eliminated, or otherwise to be inactivated, or to be rendered sensitive and/or vulnerable to destruction; (b) a sensitizing gene promoter; (c) at least one control gene; and (d) a control gene expression regulatory system responsive in use in a transfected cell to the effect of a predetermined exogenous or endogenous expression inducing influence, e.g. ionizing radiation, heat or a chemical inducing agent, so as to induce expression of the control gene to yield an expression product having a capacity to establish an operative linkage between the sensitizing gene promoter and the sensitizing gene or genes effective to trigger and switch on or permit continuous or permanent expression of the latter to bring about continuous production of the sensitizing gene expression product. This is preferably achieved by arranging for the control gene to encode a recombinase enzyme that acts on recombinase target sites in a Cre-loxP or Flp-frt site specific recombination system to remove an expression preventing stop cassette sequence between the sensitizing gene(s) and the promoter for the latter. In some embodiments the tumour sensitizing gene expression product will be an enzyme or other bioactive agent that can activate an inactive prodrug.</p>		
<b>(57) Abrégé</b> <p>L'invention concerne un vecteur présentant une grande utilité pour une thérapie antitumorale. Ce vecteur comprend (a) un gène de sensibilisation aux cellules tumorales ou des gènes dont l'expression dans une cellule tumorale produit un produit d'expression de gène de sensibilisation présentant le potentiel de tuer et de détruire ou d'éliminer les cellules tumorales, ou encore d'inactiver ces dernières ou de les rendre sensibles et/ou vulnérables à la destruction; (b) un promoteur de gène de sensibilisation; (c) au moins un gène régulateur; et (d) un système de régulation de l'expression du gène régulateur agissant lors de son utilisation dans une cellule transfectée en réponse à une expression endogène ou exogène prédéterminée induisant, par exemple, un rayonnement ionisant, un effet thermique ou un agent inducteur chimique, de manière à induire l'expression du gène régulateur pour produire un produit d'expression pouvant établir une liaison opérationnelle entre le promoteur de gène de sensibilisation et le gène de sensibilisation ou les gènes capables de déclencher ou de permettre l'expression permanente ou continue de ces derniers pour déclencher la production continue du produit d'expression du gène de sensibilisation. Cela est assuré, de préférence, en permettant au gène régulateur de coder une recombinase, enzyme qui agit sur des sites cibles de la recombinase dans un système de recombinaison ciblé pour le Flp-frt ou Cre-loxP pour retirer une séquence d'arrêt de l'expression entre le gène ou les gènes de sensibilisation et le promoteur de ce dernier. Dans certains modes de réalisation, le produit d'expression du gène de sensibilisation aux tumeurs peut être une enzyme ou un autre agent biologique actif qui permet d'activer un médicament inactif.</p>		

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/85, 15/52, 15/53, 9/00, 9/02, 15/86, A61K 48/00, A61P 35/00		A3	(11) International Publication Number: WO 99/60142
			(43) International Publication Date: 25 November 1999 (25.11.99)
(21) International Application Number: PCT/GB99/01362		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 17 May 1999 (17.05.99)		Published With international search report.	
(30) Priority Data: 9810423.5 15 May 1998 (15.05.98) GB		(88) Date of publication of the international search report: 13 July 2000 (13.07.00)	
(71) Applicant (for all designated States except US): CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [GB/GB]; Cambridge House, 6-10 Cambridge Terrace, Regent's Park, London NW1 4JL (GB).			
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(74) Agent: WILSON GUNN SKERRETT; Charles House, 148/9 Great Charles Street, Birmingham B3 3HT (GB).			
(54) Title: GENE THERAPY VECTORS AND THEIR USE IN ANTITUMOUR THERAPY			
(57) Abstract			
<p>Vector material useful for antitumour therapy contains: (a) a tumour cell sensitizing gene or genes of which expression in a tumour cell yields a sensitizing gene expression product having a potential to cause tumour cells to be killed and destroyed, or to be eliminated, or otherwise to be inactivated, or to be rendered sensitive and/or vulnerable to destruction; (b) a sensitizing gene promoter; (c) at least one control gene; and (d) a control gene expression regulatory system responsive in use in a transfected cell to the effect of a predetermined exogenous or endogenous expression inducing influence, e.g. ionizing radiation, heat or a chemical inducing agent, so as to induce expression of the control gene to yield an expression product having a capacity to establish an operative linkage between the sensitizing gene promoter and the sensitizing gene or genes effective to trigger and switch on or permit continuous or permanent expression of the latter to bring about continuous production of the sensitizing gene expression product. This is preferably achieved by arranging for the control gene to encode a recombinase enzyme that acts on recombinase target sites in a Cre-loxP or Flp-frt site specific recombination system to remove an expression preventing stop cassette sequence between the sensitizing gene(s) and the promoter for the latter. In some embodiments the tumour sensitizing gene expression product will be an enzyme or other bioactive agent that can activate an inactive prodrug.</p>			

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# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/GB 99/01362

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/85 A61K48/00 C12N15/52 C12N15/53 C12N9/00  
C12N9/02 C12N15/86 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A01K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GU, H. ET AL.: "Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting" SCIENCE., vol. 265, 1 July 1994 (1994-07-01), pages 103-106, XP000857325	1-3,5,6, 13,14
Y	AAAS. LANCASTER, PA., US page 104, column 1, paragraph 2 -column 3, paragraph 2; figure 1	1-9,11, 13-18, 23-25, 27,28, 36,41
	page 105, column 2, paragraph 3 -column 3, paragraph 2	
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☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/GB 99/01362

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 195 30 412 A (MELCHNER HARALD VON PROF DR ;GREZ MANUEL DR (DE); RUSS ANDREAS PET) 20 February 1997 (1997-02-20)	1,2,5,6, 8,14-18
Y	the whole document	1-4,7,9, 13-18, 23-25, 27,28,41
X	ANTON M ET AL: "SITE-SPECIFIC RECOMBINATION MEDIATED BY AN ADENOVIRUS VECTOR EXPRESSING THE CRE RECOMBINASE PROTEIN: A MOLECULAR SWITCH FOR CONTROL OF GENE EXPRESSION" JOURNAL OF VIROLOGY,US,THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 69, no. 8, August 1995 (1995-08), pages 4600-4606-4606, XP002011775 ISSN: 0022-538X cited in the application	1,2,5,6, 8,14
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Inter. Appl. Application No.  
PCT/GB 99/01362

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>FERNEX, C. ET AL: "Cre/loxP mediated excision of a neomycin resistance expression unit from an integrated retroviral virus increases Long Terminal Repeat-driven transcription in human hematopoietic cells"</p> <p>JOURNAL OF VIROLOGY., vol. 71, no. 10, October 1997 (1997-10), pages 7533-7540, XP000857099</p>	1,4,5
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Y	<p>HALLAHAN, D.E. ET AL.: " Spatial and temporal control of gene therapy using ionizing radiation"</p> <p>NATURE MEDICINE, vol. 1, no. 8, August 1995 (1995-08), pages 786-791, XP000857200 cited in the application the whole document</p>	1,4,11, 16, 23-25, 27,28
Y	<p>HALLAHAN, D.E. ET AL.: "c-jun and Egr-1 participate in DNA synthesis and cell survival in response to ionizing radiation exposure"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol. 270, no. 51, 22 December 1995 (1995-12-22), pages 30303-30309, XP000857098 MD US cited in the application the whole document</p>	23,27,28
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International Application No.  
PCT/GB 99/01362

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAKSO M ET AL: "EFFICIENT IN VIVO MANIPULATION OF MOUSE GENOMIC SEQUENCES AT THE ZYGOTE STAGE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 93, no. 12, June 1996 (1996-06), page 5860-5865 XP000670222 ISSN: 0027-8424 the whole document	1,5,6, 11,14
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